# EFFECT OF ACUTE AND CHRONIC AMPHETAMINE INTOXICATION ON BRAIN CHEMISTRY AND BEHAVIOR

by

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# ABSTRACT

The effects of acute and chronic *d*-amphetamine intoxication on behavior and on some neurochemical parameters were examined in mice and rats. By comparing the effects of *d*-amphetamine and *dℓ*-para-methoxyamphetamine on motor activity and the release and reuptake of brain norepinephrine (NE) and dopamine (DA), it was found that the animal's behavior in response to *d*-amphetamine administration is determined by the DA to NE receptor activity ratio and that both DA and NE neurons must be activated in order for *d*-amphetamine to induce increased exploratory activities. The effect of moderate doses of *d*-amphetamine on noradrenergic neurons (and probably dopaminergic neurons as well) was found to be self-regulating. Initially, *d*-amphetamine releases newly synthesized catecholamines and blocks their reuptake. Subsequently, as the intrasynaptic levels of free catecholamines rise, synthesis is inhibited such that the total amount of newly synthesized catecholamine released is decreased to control levels.

After having established in at least general terms the acute mechanism of *d*-amphetamine action, the effects of chronic amphetamine intoxication on behavior and brain chemistry were examined. The *d*-amphetamine metabolite and weak sympathomimetic, p-hydroxynorephedrine (PONE), accumulates in the rat brain during chronic amphetamine administration. When 10 μg of PONE is administered intracisternally, the PONE decreases NE receptor activity by competing with NE at the receptor rather than by influencing NE synthesis and metabolism. By decreasing NE receptor activity, the DA to NE receptor activity ratio is increased and, thus, challenging doses of *d*-amphetamine produced stereotyped behaviors. Amphetamine intoxication in mice and rats enhanced dopaminergic neuron activity by either increasing DA synthesis (mice) or release (rats). In conclusion, amphetamine intoxication increases the DA to NE receptor activity ratio.

Chronic amphetamine administration produces a persistent increase in spontaneous NE release which lasts for at least one week after drug withdrawal. Studies were conducted to determine what synaptic membrane constituents may be involved in the phenomenon. Data are presented which suggest that a decrease in phosphatidylcholine turnover would increase NE release. In most subcellular fractions, *d*-amphetamine inhibited phosphatidylcholine turnover. However, in the synaptic membrane fractions, *d*-amphetamine slightly enhanced phosphatidylcholine turnover. Thus, the mechanism by which *d*-amphetamine increases NE release does not appear to involve a modification in phosphatidylcholine turnover.

# CHAPTER 1

# INTRODUCTION

## 1.1 ORGANIZATION OF THESIS

The problems examined in the following thesis are all related to the general theme of what are the effects of chronic amphetamine intoxication on brain chemistry and behavior. The nature of the thesis is such that not one but several specific problems were examined. For the sake of clarity, the results have been presented in chapter form with each specific problem being discussed separately. At the end of Chapter 3, the Methods chapter, the important results obtained in Chapters 4 to 11 are briefly discussed. This will provide the reader with a synopsis of the important points I wish to emphasize.

There were two general types of problems investigated. The first, reported in Chapters 4 to 7, deals with the effects of amphetamine intoxication on behavior and the brain catecholamines. Based on the data obtained in these chapers, it appears that chronic amphetamine administration produces some permanent alterations in membrane constituents and that these alterations influence catecholamine function. In Chapters 8 to 11 the possible nature, significance and function of these changes in membrane constituents are investigated. Special emphasis is placed on the effects of amphetamine in the brain phospholipids (Chapters 9 and 10).

Background material for Chapters 4 to 11 is presented in Chapter 2. This material, though, is somewhat general in nature and was inserted primarily to serve as reference material for the reader unfamiliar with the subject. Each chapter contains sufficient historical material for the reader familiar with basic amphetamine pharmacology and CNS neurochemistry.

The methods employed in this thesis are discussed in Chapter 3. If the reader once familiarizes himself with these methods, there is sufficient information in the individual chapters to serve as a reminder of the methodological details. Special experimental procedures, e.g., animals, drug dosage, etc., are presented in the individual chapters.

In conclusion, the chapter technique has certain advantages and disadvantages. The major advantage is that it allows each problem examined to be seen clearly and concisely. However, this advantage is also the major disadvantage since there is a sacrifice of continuity. This is a dilemma for which I have no solution and I apologize to the reader who finds the disadvantages greater than the advantages.

## 1.2 THE PROBLEM

The purpose of this thesis is to develop a working hypothesis derived from experimental evidence which will explain the gross behavioral alterations which occur during chronic amphetamine intoxication. Clinically, chronic amphetamine intoxication produces symptoms of paranoid schizophrenia, depression, and excessive stereotyped activities, to name a few signs (Kalant, 1966). Surprisingly, there have been only a few studies concerned with the neurochemical mechanisms underlying these signs.

The bulk of the existing studies on the behavioral and chemical effects of amphetamine have been concerned with the acute drug effects. The data suggest that amphetamine-induced behaviors result primarily from the potentiation of the action of the brain catecholamines by blocking catecholamine uptake and increasing catecholamine release (see the review by Sulser and Sanders-Bush, 1969). Weissman *et al.* (1966) and subsequently others (Hanson, 1967; Sulser *et al.*, 1968; Randrup and Munkvad, 1966) have suggested that *d*-amphetamine acts primarily on a small pool of newly-synthesized and physiologically active catecholamines. Wise and Stein (1970) have questioned the notion that the catecholamines must be newly synthesized and have proposed that catecholamines could be provided exogenously as long as they were sequestered in a mobile pool within the nerve terminal. However, the work of Sedvall *et al.* (1968) suggests that under normal conditions the small mobile pool is composed of newly synthesized catecholamines.

Regardless of whether one adheres to the multicompartmental model alluded to above or if one adheres to the single open compartmental model of the adrenergic neuron proposed by Costa and co-workers (see Neff *et al.*, 1971), the demonstration that tyrosine hydroxylase inhibitors block at least some of the centrally mediated *d*-amphetamine-induced behaviors such as increased locomotor activity and stereotyped behaviors prompted many investigators to examine the effect of *d*-amphetamine on brain catecholamine synthesis *in vivo* (Lewander, 1970; Costa and Groppetti, 1970; Glowinski, 1970; Beeson *et al.*, 1969, 1971; Javoy *et al.*, 1968; Persson, 1970). Perhaps as a result of methodological differences, the results of these experiments have been conflicting and, consequently, the precise effects of *d*-amphetamine on the synthesis and turnover of newly formed catecholamines are not known.

Another disturbing aspect of the theories about the centrally mediated behaviors caused by *d*-amphetamine concerns the relative roles brain norepinephrine (NE) and dopamine (DA) exert in particular behaviors. In 1966 Randrup and Scheel-Krüger suggested that the potentiation of the action of brain NE caused by *d*-amphetamine results in increased locomotor activity, while the potentiation of the action of brain DA (primarily in the neostriatum) results in the development of stereotyped behaviors. This fairly simplistic theory persisted for several years and, in regards to the development of stereotyped behaviors, the dominant role of brain DA is still held to be true today. However, the role of brain NE in the increased locomotor activity has come under attack. Chemical (Costa and Groppetti, 1970; Persson, 1970) and behavioral (Ahlenius and Engel, 1971; Fuxe and Ugerstedt, 1970) studies have suggested that brain DA may play a more prominent role in the increased locomotor activities than was originally expected. This conclusion, though, is still controversial (Chan and Webster, 1971; Bizzi *et al.*, 1970).

Thus, it would appear that there are at least two problems concerning the acute pharmacology of *d*-amphetamine that must be resolved before proceeding to an examination of the acute drug effects. Briefly stated, these problems are (1) in the context of a consistent methodological approach, is it possible to show that *d*-amphetamine has a similar effect on brain catecholamine synthesis and turnover using both *in vivo* and *in vitro* techniques, and (2) is it possible to determine the role brain DA plays in the *d*-amphetamine induced increase in locomotor activity. Experiments designed to answer these questions are presented in Chapters 4 and 5.

Acute and chronic amphetamine intoxication is known to produce severe personality disturbances. These include complex visual and olfactory hallucinations, depersonalization, hypersexuality, paranoid schizophrenia, withdrawal depression and abnormal sleeping patterns and complex stereotyped behaviors (Kalant, 1966; Ashcroft *et al.*, 1965; Connell, 1966; Matteson and Calverly, 1968; Randrup and Munkvad, 1967, 1970; Kramer *et al.*, 1967; Oswald and Thacore, 1963; Griffith *et al.*, 1970). Some workers feel that amphetamine-induced psychosis may serve as one of our best, if not our only, pharmacological models of functional psychosis (Connell, 1958; Bell, 1965; Kety, 1967; Domino, 1969). Outside of the numerous clinical reports describing the effects of acute and chronic amphetamine abuse (see Kalant, 1966; Connell, 1958, 1966), there have been few well-designed studies on the behavioral and chemical effects of repeated amphetamine administration in either animals or man. Repeated injections of amphetamine have been found to produce tolerance to some centrally mediated amphetamine behaviors (Schuster and Zimmerman, 1961; Fischman and Schuster, 1974; Schuster *et al.*, 1966; Harrison *et al.*, 1952) but not to others (Brodie *et al.*, 1969; Schuster *et al.*, 1966). Gunne and Lewander (1968) and Lewander (1968) have examined the effect of both short and long-term chronic amphetamine treatment on heart, brain and urinary catecholamine levels in the rat.

The problems associated with studying the behavioral-chemical aspects of chronic amphetamine intoxication are complicated in certain species, notably the rat, by the presence of the *d*-amphetamine metabolite and false transmitter *p*-hydroxynorephedrine (PONE). PONE is a weak sympathomimetic (Brodie *et al.*, 1969) which displaces endogenous NE, is sequestered in the storage vesicles and can be released by successive doses of *d*-amphetamine, reserpine or electrical stimulation (Brodie *et al.*, 1969, 1970; Costa and Groppetti, 1970; Fischer *et al.*, 1965; Gill *et al.*, 1967). The fact that PONE can be released in a variety of ways similar to endogenous NE has suggested to Costa and Groppetti

(1970) that the presence and release of this substance in chronic amphetamine abusers may contribute to their depression and psychotic reactions.

The effects of PONE per se on brain NE synthesis and turnover and on amphetamine induced behaviors is not known. Kopin *et al.* (1968) have shown that false transmitters similar in structure to PONE depress catecholamine synthesis in the rat heart at both the level of tyrosine hydroxylase and dopamine-β-hydroxylase. If a similar effect occurs in regards to brain NE synthesis, one would predict that such an effect would contribute further to the depression associated with chronic amphetamine use and would act to block the effectiveness of *d*-amphetamine in releasing newly-synthesized catecholamines.

Thus, the first problem concerning chronic amphetamine intoxication to be examined in this thesis deals with an investigation of the effects of PONE per se. Experiments designed to answer the questions of what is the effect of PONE on catecholamine synthesis and turnover and of how does PONE effect the actions of challenging doses of *d*-amphetamine on behavior and the brain catecholamines are presented in Chapter 5.

It is likely that chronic (or acute) amphetamine intoxication will produce alterations in catecholamine synthesis, metabolism or function which are independent of the accumulation of PONE. Javoy *et al.* (1968) have found that chronic (4 days) treatment with twice daily injections of 5 mg/kg i.p. of *d*-amphetamine did not change NE turnover in two regions of the rat brain. Gunne and Lewander (1968) have noted, however, that the initial increase in urinary catecholamine levels caused by repeated amphetamine injections begins to return to normal after several days, indicating that some tolerance has developed. It is difficult, though, to extrapolate these data to changes in brain catecholamine metabolism, since the urinary catecholamines are primarily derived from peripheral stores. Fuxe *et al.* (1970) have observed with histochemical techniques that the chronic (2 to 6 weeks) injection of large doses of *d*-amphetamine (20 to 30 mg/kg i.p.) to rats causes a reduction in brain NE levels which persists till at least 48 hours after the last injection. Outside of the above mentioned studies, there are no substantial reports on the effect of chronic amphetamine intoxication on the brain catecholamines. Some data have been alluded to in one monograph (Fuxe and Ugerstedt, 1970) and some data have been reported to this author orally (personal communication from Lewander, 1974), but has yet to appear in a regular publication. Considering the marked behavioral effects caused by chronic amphetamine treatment, the paucity of chemical information on this subject seems somewhat unusual. There are a number of questions concerning the effects of chronic amphetamine treatment which come to mind.

For example, what is the effect of chronic amphetamine treatment on catecholamine uptake, release and synthesis? What are the behavioral effects of repeated amphetamine administration and can any changes that are observed be correlated with alterations in the brain catecholamines? These questions are dealt with in Chapters 6 and 7.

Oswald and Thacore (1963) have observed that amphetamine and other drugs suppress paradoxical sleep, tolerance develops to this effect, and upon drug withdrawal, paradoxical sleep increases in duration. Readministration of the drug abolishes this abstinence syndrome. Kramer *et al.* (1967) have suggested that the exaggerated sleepiness following the withdrawal of amphetamine in a chronic abuser is a sign of physical dependence. These authors further stated that "the depth and length of the withdrawal sleep and the fatigue which lasts for several weeks afterward cannot be explained by chronic fatigue alone, but that physiological-biochemical adaptations occur with chronic use of amphetamines which are analogous to those changes which are hypothesized to explain the abstinence syndrome due to the opioids or barbiturates". Similarly, Oswald (1970) has suggested that the REM rebound which occurs after the withdrawal of amphetamine "takes many weeks to disappear and just as tolerance to some drugs appears to require protein synthesis in cerebral neurons (e.g., Way *et al.*, 1968), so the withdrawal rebound, and the very slow return of brain function to normal that it reveals, indicate that protein synthesis is required for the reformation of neuronal machinery that became modified while the drug was being taken." A more general interpretation of Oswald's hypothesis is that during chronic amphetamine treatment the drug could alter a variety of slowly turning over molecules. These molecules need not be proteins but could be, for example, phospholipids, glycolipids or glycoproteins as well. Alterations in any of these substances could affect the function of the putative neurotransmitters including the catecholamines which are involved in REM sleep (Jouvet, 1969).

Data presented in Chapter 7 suggest that chronic amphetamine intoxication causes "permanent" alterations in catecholamine function. The possibility that these alterations are related to modifications in membrane constituents is investigated in Chapters 8 to 11. In Chapter 8 the question asked is what role do the various membrane constituents play in catecholamine release and reuptake? Based on the data obtained in Chapter 8, Chapters 9 and 10 deal with an investigation of the effects of chronic amphetamine treatment on brain phosphatidylcholine.

Chapter 11 is concerned with the effect of amphetamine on brain protein synthesis and turnover.

The bulk of the work described in this thesis was done between 1971 and 1973. Since the thesis itself was written over a year later, there is the additional advantage of hindsight and more laboratory experience from which the data can be analyzed. In truth, knowledge of new methods and recent publications would cause me to redesign several of the experiments if they were done today. Therefore, I have included some additional discussion in the relevant chapters outlining present criticisms of the techniques and/or the experimental design used and proposing different and perhaps more viable approaches.

# CHAPTER 2

# HISTORICAL PERSPECTIVE

## 2.1 CATECHOLAMINES - INTRODUCTION.

Although the behavioral effects of *d*-amphetamine undoubtedly arise out of complex interactions between many neurotransmitters, it is clear that the catecholamines play a dominant role in the acute and chronic drug-induced behaviors. For this reason, it would seem prudent to review the synthesis, metabolism, disposition and probable function(s) of catecholamines in the CNS.

## 2.2 CATECHOLAMINE SYNTHESIS.

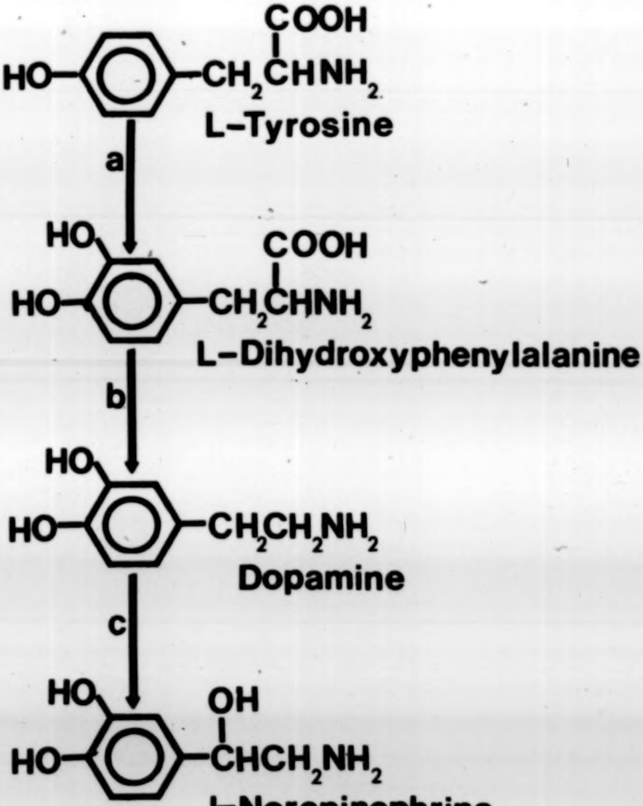
Catecholamines are synthesized from L-tyrosine by a pathway shown in Fig. 1 and first described by Blaschko (1939). The characterization of this pathway initially came from studies using the adrenal medulla (Demis *et al.*, 1956; Udenfriend and Wyngarden, 1956; Pellerin and D'Iorio, 1957; Hagen, 1956) and subsequently other tissues, including the brain (Goodall and Kirshner, 1956; Masuoka *et al.*, 1963; Musacchio and Goldstein, 1963; Spector *et al.*, 1963).

## 2.3 TYROSINE HYDROXYLASE.

The rate limiting enzyme in catecholamine formation is tyrosine hydroxylase. The characteristics of the adrenal enzyme have been best described, although the brain enzyme does not appear to be substantially different (Nagatsu *et al.*, 1964; Udenfriend, 1966; Ikeda *et al.*, 1965; Bagchi and McGeer, 1965; McGeer *et al.*, 1965; McGeer and McGeer, 1967). There appears to be sufficient supply of L-tyrosine to saturate

Fig. 1. Biosynthetic pathway for catecholamines.

a = Tyrosine hydroxylase, b = Aromatic amino acid decarboxylase, c = Dopamine-β-hydroxylase.


L-Tyrosine
a
L-Dihydroxyphenylalanine
b
Dopamine
c
l-Norepinephrine

the available enzyme (Udenfriend, 1966). The $K_m$ for the conversion of L-tyrosine to L-dihydroxyphenylalanine (DOPA) is $2 \times 10^{-5}$ M while the tyrosine levels in the plasma and other tissues are in the order of $10^{-4}$ M. Increasing the tyrosine levels *in vivo* does not result in increased levels of DA and NE (Udenfriend, 1966).

Tyrosine hydroxylase is a mixed function oxidase which requires a ferrous salt and the endogenous cofactor dihydrobiopterin or the synthetic cofactor tetrahydrobiopterin. The iron requirement of the enzyme has been the basis of one report which suggests that catecholamine turnover can be measured by inhibiting tyrosine hydroxylase with the chelating agent α,α-dipyridyl (McGeer and McGeer, 1967). Perhaps the most familiar inhibitor is α-methyl tyrosine (α-MT) (Udenfriend *et al.*, 1965; Spector *et al.*, 1966). Only a small proportion of the α-MT is converted to α-methyl DOPA (Maitre, 1965). However, a recent report by Doteuchi *et al.* (1974) indicated that a significant portion of α-MT is converted to p-OH-amphetamine (PA) and subsequently p-OH-norephedrine (PONE). The accumulation of PA and PONE in adrenergic neurons was demonstrated to in part be responsible for the α-MT induced depletion of catecholamines. This finding may be of additional importance in understanding the effects of α-MT on amphetamine-induced behaviors.

Tyrosine hydroxylase is located both in the cell body and nerve ending (synaptosome) of the adrenergic neuron. Presumably, the site of enzyme synthesis is the endoplasmic reticulum of the perikaryon and the enzyme is then transferred to the nerve ending via axoplasmic flow (Grafstein, 1969). Laudron (1968) and Laudron and Belpair (1968a,b) have provided evidence that in the synaptosome tyrosine hydroxylase is located in the synaptosomal cytoplasm and is not associated with the synaptic vesicles.

Since tyrosine hydroxylase is the rate limiting enzyme in catecholamine formation, those factors which govern the rate of catecholamine synthesis *in vivo* and *in vitro* are generally associated with a change in hydroxylase activity. This area has been discussed in detail by Weiner (1970). Briefly, it appears that the principal factor which regulates tyrosine hydroxylase activity is the intrasynaptic level of unbound catecholamines. Unbound catecholamine levels are influenced by nerve activity (Roth *et al.*, 1966; Alousi and Weiner, 1966) or drugs (Weiner *et al.*, 1972). The free catecholamines apparently compete with the pteridine cofactor for the oxidized enzyme (Ikeda *et al.*, 1966; Weiner *et al.*, 1972; Udenfriend *et al.*, 1965). Finally, the importance of pteridine in regulating tyrosine hydroxylase activity has suggested to some workers that the activity of the dihydrofolate reductase may be the rate limiting enzyme in catecholamine synthesis (Musacchio, 1972).

## 2.4 DIHYDROXYPHENYLALANINE DECARBOXYLASE

The second step in catecholamine formation from L-tyrosine involves the decarboxylation of L-dihydroxyphenylalanine (DOPA) to DA. DOPA decarboxylase is generally referred to as the aromatic amino acid decarboxylase since it is capable of decarboxylating a number of substrates including 5-hydroxytryptophan (5-HTP) and histidine (Lovenberg *et al.*, 1962). As with other decarboxylases, DOPA decarboxylase requires pyridoxal-5'-phosphate (PLP) for enzyme activity. Unlike histidine decarboxylase, PLP appears to be tightly bound to the enzyme

(Hakanson, 1967; Udenfriend, 1964). The highest level of decarboxylase activity in the rat brain is found in the caudate nucleus; fairly high levels have been reported in the medulla, the thalamus and superior colliculus and low values in the cortex (McCaman and Aprison, 1964). Decarboxylase activity is found in glial cells; the enzyme is thought to play an important role in the blood-brain barrier which prevents DOPA (or 5-HTP) from gaining access to the brain (Owman and Rosengren, 1967). Although there are many decarboxylase inhibitors, one of the most widely used is 3-hydroxy-4-bromobenzyloxyamine (NSD-1055) (Aures *et al.*, 1970). This compound is routinely used in tyrosine hydroxylase assays (McGeer and McGeer, 1967).

## 2.5 DOPAMINE-β-HYDROXYLASE.

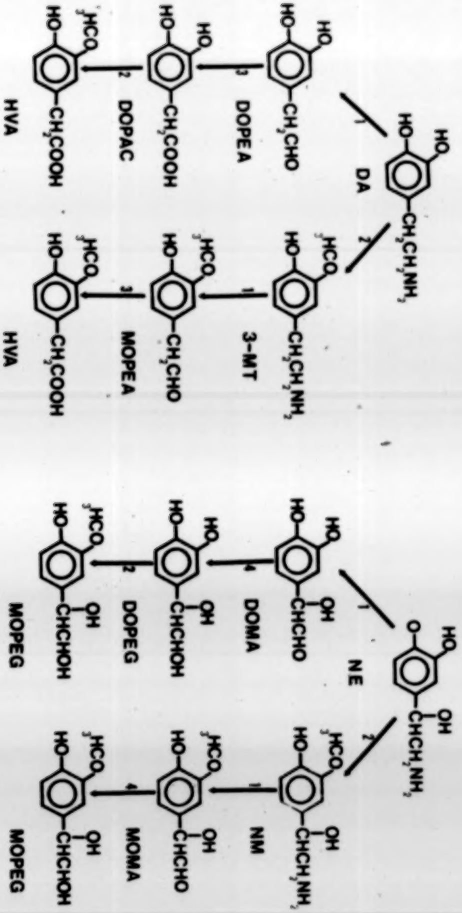
The final enzyme in NE synthesis is dopamine-β-hydroxylase (DA-β-hydroxylase). This enzyme is the only one in the catecholamine synthetic sequence which has been highly purified (Kaufman and Friedman, 1966). Unlike tyrosine hydroxylase and DOPA decarboxylase, DA-β-hydroxylase is not a "soluble" enzyme but is firmly attached to the storage vesicle membranes (Duch *et al.*, 1968; Viveros *et al.*, 1969). Certain lines of pharmacological evidence suggest that the transport of DA through the vesicular membrane is coupled with hydroxylation (Rutledge and Weiner, 1967; Kopin *et al.*, 1968).

Dopamine-β-hydroxylase purified from the bovine adrenal medulla is a copper containing enzyme with an apparent molecular weight of 290,000 (Kaufman and Friedman, 1966). The enzyme requires, in addition to copper, ascorbate and perhaps some dicarboxylic acid (Kaufman and Friedman, 1966; Goldstein, 1956). For our purposes, one of the most interesting aspects of this enzyme is its ability to hydroxylate a large number of phenethylamine substrates including *d*-amphetamine and *d*-p-OH-amphetamine (van der Shoot and Creveling, 1965; Goldstein and Anagnoste, 1965). The conversion of the latter compound to *p*-hydroxynorephedrine (PONE), a false transmitter (Kopin *et al.*, 1965), may be of importance in understanding amphetamine tolerance and intoxication (Brodie *et al.*, 1970; Costa and Groppetti, 1970). Inhibitors of DA-β-hydroxylase which have been popular in pharmacological studies include diethyldithiocarbamate (Randrup and Scheel-Krüger, 1966).

## 2.6 CATECHOLAMINE METABOLISM.

The major metabolic pathways of DA and NE in the brain are illustrated in Fig. 2. The metabolic pathways in peripheral systems and the brain were precisely elucidated soon after high specific activity $^3$H-DA and $^3$H-NE became commercially available. The metabolism of catecholamines was primarily determined by Axelrod and his co-workers (Axelrod *et al.*, 1958; LaBrosse *et al.*, 1961; Kopin *et al.*, 1961; Glowinski *et al.*, 1965). These authors found that the major pathway for metabolism of circulating catecholamines involves first 3-0-methylation catalyzed by catechol-0-methyltransferase (COMT); secondly, oxidative deamination of the methoxylated metabolites to the corresponding aldehydes catalyzed by monoamine oxidase (MAO) and, thirdly, oxidation of the aldehydes to the corresponding acids catalyzed by an aldehyde reductase. Metabolism in the brain differs from that in the periphery in that in the brain the final product of NE metabolism is generally a glycol (3,4,dihydroxyphenylglycol (DOPEG) or 3-methoxy-4-hydroxyphenylglycol (MOPEG or MHPG). Unlike NE, in the brain DA is primarily converted to 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid (HVA) or 3,4,dihydroxyphenylacetic acid

Fig. 2. Major pathways of DA and NE metabolism in the brain. Abbreviations used: DA = Dopamine 3-MT-3-Methoxytyramine; DOPEA = Dihydroxyphenethylaldehyde; DOPAC = Dihydroxyphenylacetic acid; HVA = Homovanillic acid; NE = Norepinephrine; DOMA = Dihydroxymandelic aldehyde; DOPEG = Dihydroxyphenylglycol; MOPEG = 3-Methoxy-4-hydroxyphenylglycol; NM = Normetanephrine; MOMA = 3-Methoxy-4-hydroxymandelic aldehyde; 1 = Monoamine oxidase; 2 = Catechol-O-methyltransferase; 3 = Aldehyde reductase; 4 = Alcohol dehydrogenase.


DA
DOPEA
DOPAC
HVA
3-MT
MOPEA
HVA
NE
DOMA
DOPEG
MOPEG
NM
MOMA
MOPEG

(DOPAC) (Rutledge and Jonason, 1967). Schandberg *et al.* (1968) have found that MOPEG in the brain is generally found as the conjugated sulfate. Recently, Meek and Neff (1972) have suggested that the accumulation of MOPEG·$SO_4$ in the brain or cerebral spinal fluid (CSF) after probenicid treatment may be used as a measure of NE turnover. Similarly, the accumulation of HVA can be used as an index of DA turnover in the caudate nucleus (Portiz *et al.*, 1968).

## 2.7 MONOAMINE OXIDASE (MAO).

As early as 1910 the ability of vertebrate tissue to oxidize tyramine was recognized (Ewins and Laudlaw, 1910). In subsequent years it was found that two classes of amine oxidases exist which are now generally referred to as diamine oxidase (DAO) and monoamine oxidase (MAO). The former enzyme differs from the latter in that it attacks diamines and histamine and is sensitive to inhibition by carbonyl reagents, e.g., semicarbazide (Zeller, 1963). In 1937 Pugh and Quastel recognized the ability of brain tissue to oxidize monoamines. Interestingly, in 1940 Mann and Quastel proposed that the central stimulant effects of amphetamine, which they found to be a MAO inhibitor, were related to a decrease in the production of aldehydes which inhibit brain respiration.

MAO is found in many tissues including the liver, the parotid and submaxillary glands, the gonads and in smooth and cardiac muscle (Quastel, 1970). In the brain MAO activity may show some regional distribution (Bogdanski *et al.*, 1957) but fairly high activity is found in all brain regions. It is important to realize that there is sufficient MAO activity in all parts of the brain to oxidize at least 1 mg of catecholamine or serotonin per hour per g of brain wet weight while the endogenous concentrations of these substances is generally less than 1 μg/g of wet brain weight. For this reason alone, one can see the importance of having catecholamines stored in vesicles protected from MAO. MAO is not only associated with sympathetic and serotonergic neurons, but is also found in glial cells (Bertler *et al.*, 1966).

MAO activity appears to be entirely localized in the mitochondria and predominantly in the outer mitochondrial membrane (Schnaitman *et al.* 1967; Tipton, 1967). Reports suggesting MAO activity can also be found in the microsomal fraction (Hawkins, 1952; Roth and Stjärne, 1966) are probably in error, with the MAO activity being associated with fragments of the mitochondrial membranes formed as an artifact during homogenization (Jarrott and Iversen, 1968). Since nerve ending particles (NEP) contain "trapped" mitochondria, MAO activity is also found in the NEP fraction (DeLores Arnaiz and DeRobertis, 1962).

Such a large number of MAO inhibitors have been found that it would be impossible to mention them all. Iproniazid, pargyline and tranylcypromine are some of the most commonly used. It is important to realize that MAO inhibitors, especially the irreversible hydrazine inhibitors, affect not only MAO but also the activity of other enzymes as well, e.g., DOPA decarboxylase and DA-β-hydroxylase (Hagen and Welch, 1956; Brodie *et al.*, 1959; Clark, 1963; Quastel, 1970). Amphetamine is an inhibitor of MAO *in vitro* (Mann and Quastel, 1939, 1940) but there is some doubt as to whether or not the drug is effective *in vivo* (Schayer *et al.*, 1954; von Euler and Hellner-Bjorkman, 1955). The mechanism by which *d*-amphetamine blocks oxidative catecholamine metabolism *in vivo* appears to principally involve inhibition of the amine reuptake mechanism (Rutledge, 1970).

## 2.8 CATECHOL-0-METHYLTRANSFERASE (COMT).

Although it may be only personal experience, no single enzyme involved in catecholamine synthesis or metabolism appears to be as misunderstood as COMT. In part, I think this results from textbook illustrations of adrenergic neurons which place COMT at some extraneuronal site close to the synaptic apparatus where it can readily metabolize excess DA or NE. From a functional viewpoint, this illustration may be correct. However, the exact cellular localization of COMT remains obscure. In peripheral tissues COMT activity is not reduced after sympathectomy, suggesting the enzyme is not located in catecholamine containing neurons (Potter *et al.*, 1965). In subcellular distribution studies the majority of COMT activity is found in the soluble fraction, although some activity is associated with NEP (Alberici *et al.*, 1965). It is possible, though, that this synaptosomal COMT is an artifact of homogenization caused by a small amount of enzyme trapped in the NEP during the shearing and sealing process. More recently, Jarrott and Iversen (1971) and Jarrott (1971) have examined the distribution of COMT before and after chemical and surgical sympathectomy. These authors have concluded that a certain portion of the total COMT activity, albeit a small portion, is located intrasynaptically. The importance of understanding that the subcellular localization of COMT is not firmly established is especially relevant to the study of *d*-amphetamine effects on NE metabolism *in vivo* and *in vitro*. Depending on the experimental design employed, *d*-amphetamine induced increases in the formation of labeled normetanephrine (NM) from labeled NE have been taken to mean that the drug increases NE release and blocks NE uptake (Glowinski *et al.*, 1965; Glowinski and

Axelrod, 1966). While the latter phenomenon can be clearly demonstrated *in vitro* (Iversen, 1967) in studies independent of COMT activity, the use of labeled NM formation as an index of *d*-amphetamine's effect on NE release should still be interpreted with some caution.

The properties of COMT have been most extensively studied using a rat liver preparation. The enzyme utilizes S-adenosyl-β-methionine as the methyl donor and has a requirement for magnesium or another divalent cation (Axelrod and Tomchick, 1958). In general, methylation occurs on the meta position, but in some cases the para position can be methylated (Senoh *et al.*, 1959). However, there is no evidence that the dimethoxy product can be formed (Daley *et al.*, 1960).

## 2.9 DISTRIBUTION OF NE AND DA IN THE CENTRAL NERVOUS SYSTEM (CNS) AND STRUCTURES ASSOCIATED WITH SYNAPTIC FUNCTION.

Although NE and DA are found in all gross regions of the brain, the distribution of these amines on a μg/g basis shows wide variations. It would seem prudent to ask if concentration of amine alone is a sufficient criteria to define a region as rich or poor in noradrenergic or dopaminergic neurons? The answer to this question is probably yes and no. Yes, in the sense that if NE and DA are detected in cell bodies and nerve endings, their presence implies adrenergic tracts. No, in the sense that turnover rather than concentration should probably be used to detect adrenergic neurons. For example, if NE is turning over very rapidly in some region but the steady-state concentration is low, it would be erroneous to conclude that this region is poor in noradrenergic innervation. Unfortunately, even the elaborate histochemical fluorescence techniques upon which much of our information concerning the localization of noradrenergic and dopaminergic innervation is derived depends primarily on concentration rather than turnover.

The main noradrenergic and dopaminergic tracts in the CNS are shown in the schematic diagram in Fig. 3 which is adapted from Andén *et al.* (1966). The distribution of brain DA and NE is also reviewed by Hillarp *et al.* (1966) and is discussed by Fuxe and Ugerstedt (1970). NE cell bodies are primarily located in the ventral brain, although small groups have been found in the hypothalamus as well. The axons from the ventral cell bodies terminate primarily in the hypothalamus, thalamus and limbic system. It is interesting to note that NE neurons are located in the phylogenetically older parts of the brain. The major DA tract originates in the pars compacta of the substantia nigra and terminates in the neostriatum. A tubero-infundibular DA system has also been demonstrated.

Although catecholamines are found in the cell bodies and axons of adrenergic tracts, the majority of the catecholamines are located in the distal portions of the axon in "varicosities". When brain tissue is homogenized, the "varicosities" are pinched off, reseal themselves and form structures identified as nerve ending particles (NEP) or synaptosomes (Whittaker, 1969; DeRobertis and deLores Arnaiz, 1969). The concentration of DA or NE in the NEP may be as high as 10,000 μg/g or $10^{-1}$ M (Hillarp *et al.*, 1966). A single neuron of the DA nigro-neostriatal system may contain 500,000 varicosities on a single axon (Andén *et al.*, 1966).

A typical NEP prepared from the rat brain cortex is shown in Fig. 4. The major structural characteristics of the NEP are a limiting plasma membrane, one or more mitochondria and synaptic vesicles. A

Fig. 3. Schematic drawing showing in highly simplified form the main monoamine neurons in the central nervous system (adapted from Andén *et al.*, 1966). Abbreviations used: NE = Norepinephrine; DA = Dopamine; Cx = Cortex; LF = Limbic Forebrain; Ns = Neostriatum; Ps = Paleostriatum; Th = Thalamus; Ht = Hypothalamus; Pt = Pituitary.

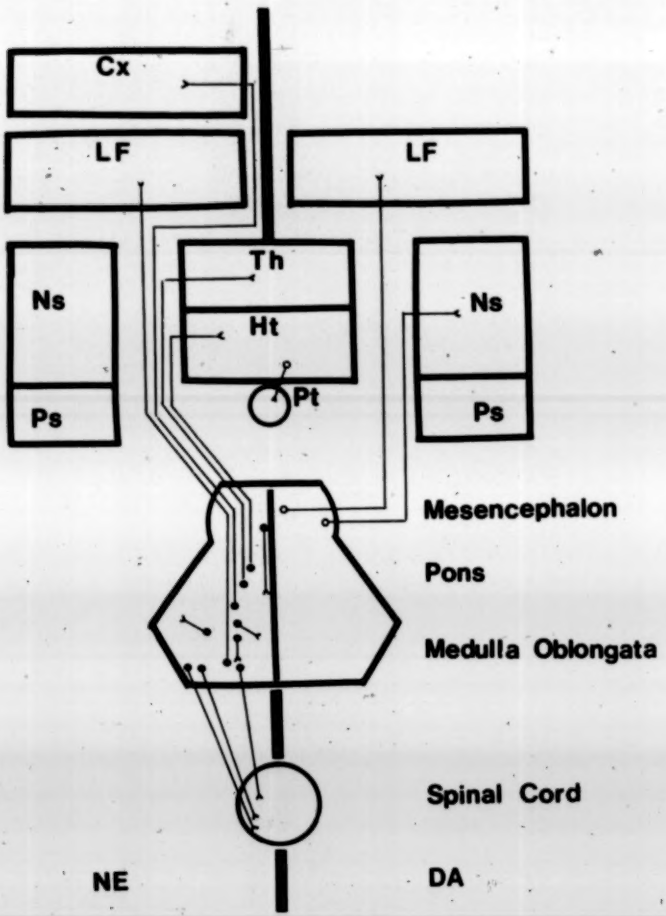
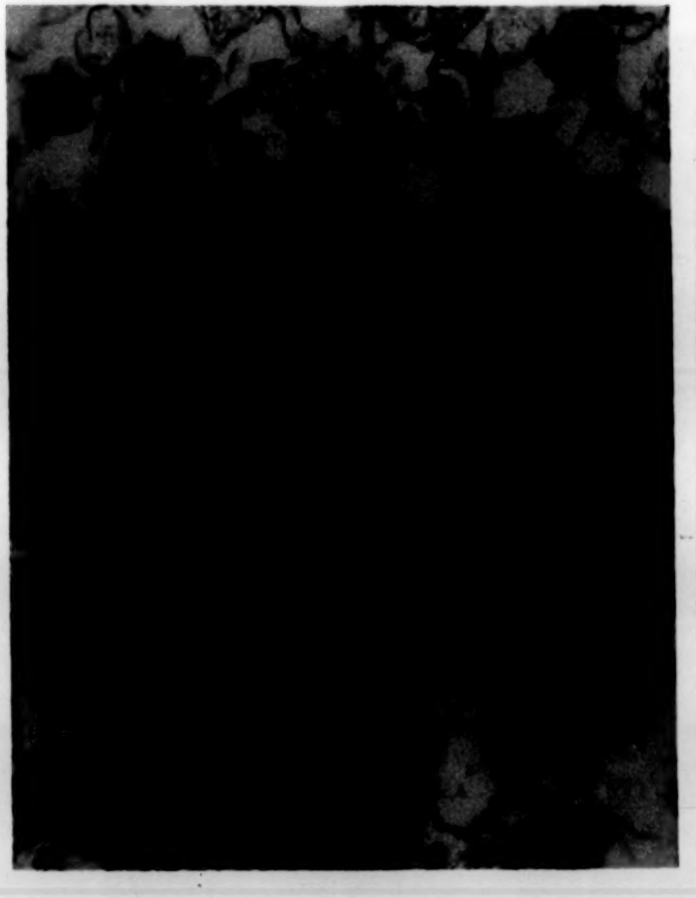

Cx
LF
LF
Th
Ht
Ns
Ns
Ps
Ps
Pt
Mesencephalon
Pons
Medulla Oblongata
Spinal Cord
NE
DA

Fig. 4. A typical NEP isolated from the rat brain cortex (method of Von Hungen *et al.*, 1968). Staining methods are given in Section 3.4. Magnification x 25,000.



schematic diagram of the synapse is shown in Fig. 5. Bloom and Aghajanian (1968, 1966) have shown that the protein of the synaptic apparatus is particularly rich in basic amino acids, i.e., lysine, arginine and histidine. The question of whether or not these basic amino acids serve a particular functional role remains to be determined. More generally, the structure-function of the synaptic apparatus and the limiting plasma membrane is not known in great detail. Lehninger (1968) has discussed possible roles of the glycoprotein-glycolipid coat which covers nerve endings and cell bodies. Fig. 6 shows his hypothetical model of the cell coat and the plasma membrane. The cell coat is located on the external surface of the plasma membrane. Lehninger has suggested that the negatively charged sialic acid heads of the glycoproteins or glycolipids extend into the intracellular space like antennae and, consequently, are exposed to extracellular $Na^+$ and $Ca^{++}$ ions. "Changes in concentration of these ions in the neighborhood of these antennae could cause conformational changes which might be transmitted mechanically to the hydrocarbon bilayer and induce local changes in its packing arrangement." Furthermore, removal of gangliosides from neuronal tissue results in the loss of electrical excitability (McIlwain, 1966). Finally, our own studies (Chapter 8) indicate that glycoproteins serve a particular role in NE uptake.

The mitochondria of the NEP apparently serve the same role in the NEP as they do in other parts of the neuron; principally, they supply the energy in the form of ATP required for a variety of neuronal processes, e.g., catecholamine storage, release and reuptake, cAMP formation, phospholipid synthesis.

Fig. 5. Structural and functional aspects of the synapse (adapted from Bloom, Iversen and Schmitt, 1971).

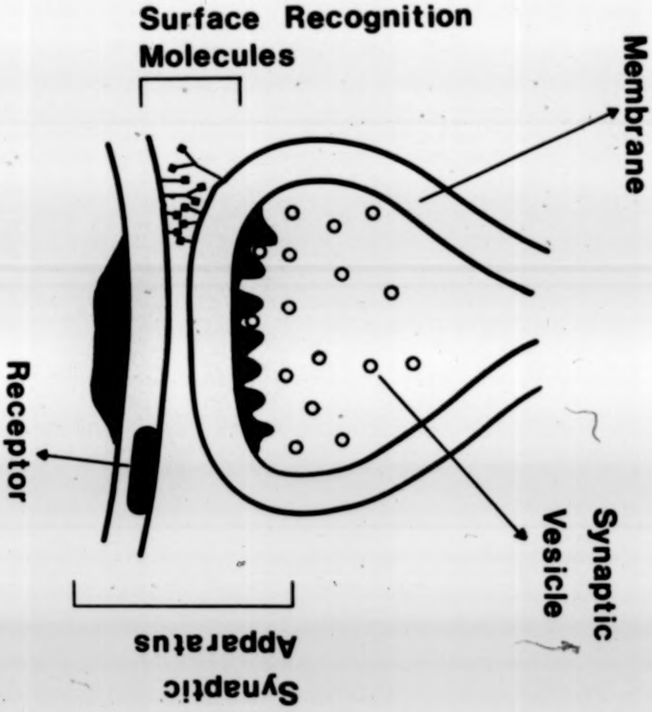
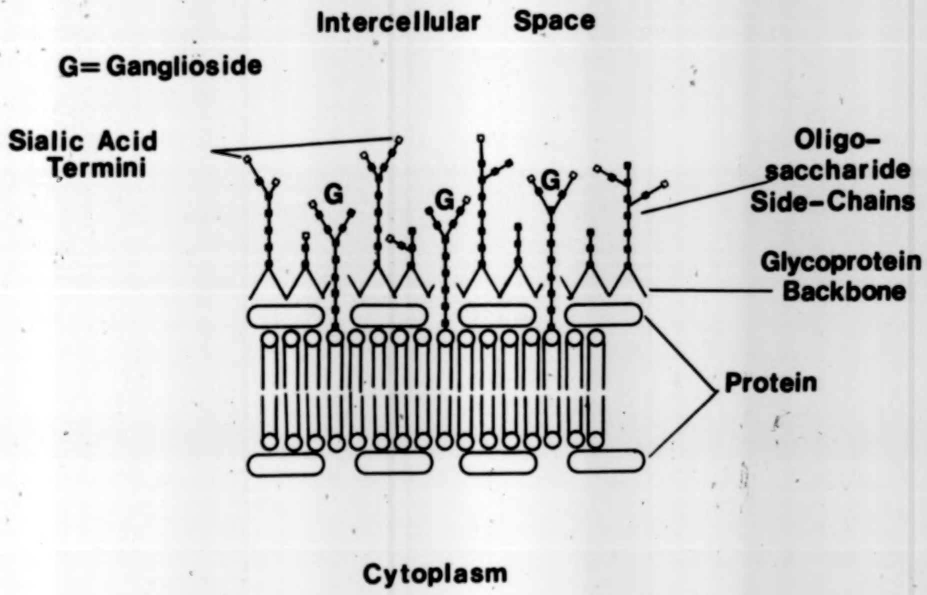

Surface Recognition Molecules
Membrane
Receptor
Synaptic Vesicle
Synaptic Apparatus

Fig. 6. Representation of the neuronal cell coat. The diagram shows the negatively charged sialic acid heads of the glycoproteins and glycolipids extending into the external milieu where they can bind with monovalent and divalent cations (from Lehninger, 1968).


Intercellular Space
G= Ganglioside
Sialic Acid Termini
G
G
G
Oligo-saccharide Side-Chains
Glycoprotein Backbone
Protein
Cytoplasm

Based on information in peripheral systems, the storage of catecholamines in the synaptic vesicles requires ATP and a protein, chromogranin A (Banks, Helle and Mayor, 1969). Pletscher *et al.* (1970) have described a biophysical model for the formation of high molecular weight aggregates of ATP and catecholamines. The formation of the aggregates depends on the presence of small amounts of $Ca^{++}$ or $Mg^{++}$. High concentrations of these divalent ions will cause the aggregates to break down. This phenomenon is of interest in light of the fact that the entry of $Ca^{++}$ into the NEP is a prerequisite for catecholamine release (Katz and Kopin, 1967). Furthermore, certain drugs, e.g., tyramine, *d*-amphetamine and reserpine, cause the aggregates to break down (Pletscher *et al.*, 1970, unpublished observation in this laboratory).

The synaptic vesicles which store the catecholamines in the CNS appear as dense core vesicles (Hökfelt, 1967). Some studies have suggested the primary storage vesicles were 800-1000 $A^{0}$ in diameter (Aghajanian and Bloom, 1967; Lenn, 1967; Bloom and Aghajanian, 1968). However, Hökfelt (1967, 1968, 1969) was able to demonstrate by using the less severe potassium permanganate fixation technique (Richardson, 1966) that the dense core vesicles in the "varicosities" of adrenergic neurons were 500 $A^{0}$ in diameter. The vesicles found in the cell perikaryon appear to be a mixture of smaller (500 $A^{0}$) and larger vesicles (800-1000 $A^{0}$) (Hökfelt, 1969).

Upon subcellular fractionation, 60 percent or more of the brain NE as well as 5-HT and ACh is found in the NEP (Glowinski and Baldessarini, 1966; Garattini and Valzelli, 1965; Whittaker, 1965) and more particularly in the synaptic vesicles. However, when the striatum is fractionated less than 40 percent of the DA is found in the particulate fraction (Glowinski and Baldessarini, 1966; O. Hornykiewicz, 1966). Various theories have been offered to explain this phenomenon (Glowinski, 1971). One possible explanation is that DA is less firmly bound to the synaptic vesicles than NE (Musacchio *et al.*, 1965).

The lipid composition of the various constituents of the NEP show wide differences as illustrated in the data in Tables 1 and 2 taken from Whittaker (1969). There are certain features of the differences in composition which deserve some special comment. It is obvious that there are some important differences between the synaptic vesicles (SV) and the limiting synaptic plasma membranes (SPM). The SPM in comparison to the SV are richer in phosphatidic acid, ethanalomine plasmalogen and ganglioside and poorer in phosphatidylcholine, phosphatidylethanalomine, phosphatidylserine, phosphatidylinositol (mono) and sphingomyelin. The molar ratio of cholesterol:phospholipid of the SPM is nearly twice that of the SV. On the basis of disc gel electrophoretic patterns, the proteins of the SV and SPM show some differences (Mahler and Cotman, 1970). Using the differences in lipid composition as his major criteria, Whittaker (1969) has suggested that the differences in membrane composition between the SPM and SV raise serious difficulties for the micropinocytosis theory of transmitter release (Whittaker, 1966a,b). In such theories it is suggested that the synaptic vesicles fuse with the SPM prior to releasing their contents. Obviously, if the membranes are sufficiently different in structure, such fusion becomes a more difficult postulate to consider. However, Whittaker's suggestion that an alternative mechanism is probably involved may be premature. It is only in the last five or six years that we have

### TABLE 1

Phospholipid Content of Synaptosomes and Their Constituent Membranes Compared with Other Brain Membranes[a,b]

| | | | | | | Myelin | |
|---|---|---|---|---|---|---|---|
| Lipid | Synaptosomes (B) | Synaptic Vesicles (D) | SPM (G) | Microsomes ($P_3$) | Mito-chondria (C) | Large($P_1$A) | Small (A) |
| Phosphatidyl-choline | 39 | 41 | 24 | 41 | 40 | 26 | 32 |
| ethanolamine | 18 | 15 | 10 | 15 | 23 | 9 | 14 |
| serine | 13 | 12 | 3 | 12 | 6 | 13 | 14 |
| Monophosphoinositide | 4 | 5 | 1 | 4 | 5 | 3 | 3 |
| Phosphatidic acid | 1 | 0 | 6 | 0 | 1 | 2 | 1 |
| Cardiolipin | 2 | 1 | 0 | 1 | 11 | 1 | 0 |
| Ethanolamine plasmalogen | 16 | 15 | 20 | 15 | 9 | 26 | 24 |
| Sphingomyelin | 5 | 11 | 6 | 8 | 4 | 12 | 7 |
| Alkylether | 2 | 3 | 4 | 2 | 2 | 4 | 2 |
| Recovery | 99 | 94 | 74 | 98 | 101 | 91 | 97 |
| Total lipids (% of dry weight) | 46 | 72 | | 60 | 32 | 80 | 74 |

[a] The results are percentages of total lipid phosphorus and relate to guinea pig brain. The large myelin fraction was the only fraction containing detectable amounts of choline and serine plasmalogens; these each accounted for 1% of the total lipid phosphorus of that fraction.

[b] Data from Whittaker, 1969.

## TABLE 2

Cholesterol, Cerebroside, and Ganglioside Contents of Synaptosomes and Their Constituent Membranes Compared with Other Brain Membranes[a]

| Fraction | Cerebrosides: phospholipids, molar ratio | Cholesterol: phospholipids, molar ratio | Ganglioside, μmoles/mg N |
|---|---|---|---|
| Synaptosomes (B) | 0.01 | 0.43 | 0.14 |
| Synaptic vesicles (D) | 0 | 0.41 | 0.07 |
| SPM (G) | 0.05 | 0.72 | 0.27 |
| Microsomes ($P_3$) | 0.09 | 0.48 | 0.14 |
| Mitochondria (C) | 0.03 | 0.15 | 0.03 |
| Myelin | | | |
| Large ($P_1$A) | 0.5 | 1.03 | 0.07 |
| Small (A) | 0.25 | 0.73 | 0.18 |

[a] Data from Whittaker, 1969.

begun to understand the structure of one mammalian membrane; namely, the erythrocyte ghost (Carraway *et al.*, 1971; Bretscher, 1973). If rejection of the "fusion" theory is based on differences in gross membrane composition, this rejection should be taken with caution; the possibility that the SPM has regions of structure identical to the SV cannot be ignored until the fine details of the relevant membrane structure and function are resolved.

## 2.10 CATECHOLAMINE RELEASE.

The mechanisms postulated for neurotransmitter, including catecholamine, release have been reviewed in an excellent essay by Bloom, Iversen and Schmitt (1971). In the following paragraphs their major points will be summarized.

In 1957 Del Castillo and Katz postulated that ACh is released from synaptic vesicles at the neuromuscular junction by exocytosis. This hypothesis was based on certain neurophysiological data, especially the discovery of the miniature endplate potential (MEPP) and the then current electron micrographs showing synaptic vesicles in the presynaptic terminal. In the subsequent years it has become evident that a great many hormones, e.g., ACTH, insulin, oxytocin, are stored in granules and are probably released by an exocytotic mechanism similar to that postulated for neurotransmitters (see Matthews, 1970).

Evidence that catecholamines are released by exocytosis comes largely from experiments using the adrenal medulla (Douglas *et al.*, 1965; Banks and Helle, 1965; Kirshner *et al.*, 1966; Blaschko *et al.*, 1967a,b; Smith and Winkler, 1967; Poisner and Trifaró, 1967). As seen in Table 3, during catecholamine release only the components inside

### TABLE 3

### Quantitative Biochemical Evidence for Exocytosis in the Adrenal Medulla

| Constituent | Ratio to Catecholamine<br>Secreted | <br>In vesicles | Units/μmole Catecholamine |
|---|---|---|---|
| Catecholamine | 1,000 | 1,000 | mμmole |
| ATP + catabolites | 175 | 256 | mμmole |
| Total chromogranins | 186 | 208 | μg |
| Chromogranin A | 82 | 98 | μg |
| DBO | 2,000 | 1,550 | unit |
| Phospholipids | 1.2 | 180 | mμmoles |
| Cholesterol | 0.9 | 102 | mμmoles |
| Lactate dehydrogenase | 0 | 0 | |
| Phenylethanolamine N-methyltransferase (PNMT) | 0 | 0 | |

Composition of soluble components of the bovine adrenal medullary storage vesicles compared with that of perfusates of the carbachol-stimulated gland.

Sources: Smith (1968) and Viveros *et al.* (1968).

the synaptic vesicle are expelled. Other components of the cellular cytoplasm, e.g., lactate dehydrogenase, which should pass through the membrane more easily than chromogranin A are not expelled (Schneider *et al.*, 1967). Thus, the most compelling evidence for exocytosis comes from biochemical data which demonstrate the unique specificity of the components released. The fact that lipids and lipoproteins are not released suggests that the vesicular membrane remains behind and, consequently, it seems unlikely that the whole vesicle is expelled during release.

Overall, the evidence which favors exocytosis as the probable mechanism of transmitter release from all neurons may be summarized.

1. Transmitters, like hormones, are stored in specialized vesicles and are not released until the appropriate stimulus is encountered. Furthermore, the vesicular membrane serves as a "sheath" which protects the contents from passive leakage and intraneuronal degradation. Exocytosis is a plausible model for release that preserves these functions.

2. The release of catecholamines and other transmitters is completely dependent on external $Ca^{++}$ and in this requirement is very similar to glandular secretions. $Na^+$ and $K^+$ ions per se are not required (Katz and Miledi, 1969); only a local depolarizing stimulus is necessary (Katz and Miledi, 1967a,b), although normally depolarization would involve $Na^+$ and $K^+$ fluxes.

3. "The release of packaged transmitter from uniformly sized synaptic vesicles provides a simple explanation for the quantal nature of transmitter release from presynaptic terminals (Katz, 1962)."

4. The release of NE is accompanied by the release of chromo-

granin A and DA-β-hydroxylase but not by other intraneuronal soluble enzymes such as DOPA decarboxylase. Exocytosis could explain this phenomena.

5. Reserpine blocks biogenic amine storage perhaps by interfering with the formation of the ATP-amine-$Mg^{++}$ complex (Pletscher *et al.*, 1970). Although amine levels may be restored to near normal with MAO inhibitors (MAOI), release is still impaired under these conditions (Potter, 1967; Malmfors, 1969). Presumably, release is impaired because the neurotransmitter is not stored in a vesicle. However, it may be wise to remember that MAOI are nonspecific drugs and are likely to exert some effects on neuronal processes unrelated to MAO inhibition.

6. The final enzyme in NE synthesis, DA-β-hydroxylase, is primarily located in this storage vesicle. According to the exocytosis model, this would conveniently deliver newly synthesized NE to the synapse.

Evidence which argues against the exocytosis model may be summarized.

1. The quantal release of neurotransmitter could occur by a mechanism other than exocytosis. For example, release could occur by a gating mechanism in the nerve membrane.

2. Although all neurotransmitters, with the possible exception of DA, are concentrated in synaptic vesicles, there is considerable evidence to indicate that a fairly large proportion (> 10%) of the total neurotransmitter store is free in the intraneuronal cytoplasm.

3. The ratio of NE to protein released from adrenergic neurons is considerably less than that found in intact vesicles.

4. The regulation of NE synthesis presumably occurs by negative feedback inhibition at the level of tyrosine hydroxylase (Ikeda *et al.*, 1966; Weiner, 1970). During nerve stimulation synthesis is increased, presumably by removal of end-product inhibition. However, if release occurs entirely from vesicles, then it is difficult to see how increasing neuronal activity could increase synthesis. This conflict may be resolved, however, if we assume that an equilibrium exists between free and bound NE, such that a decrease in bound NE also decreases the free levels.

5. Some evidence suggests that newly synthesized catecholamines are released during nerve stimulation (Kopin *et al.*, 1968; Sedvall *et al.*, 1968). This phenomena has been kinetically interpreted to mean that NE is released from a small free intraneuronal pool. However, these data can be interpreted in a number of different ways. The vesicles at the synaptic cleft which partially release their contents during neuronal transmission may be the first vesicles to fill with newly synthesized transmitter. Such a phenomenon would explain the observed results.

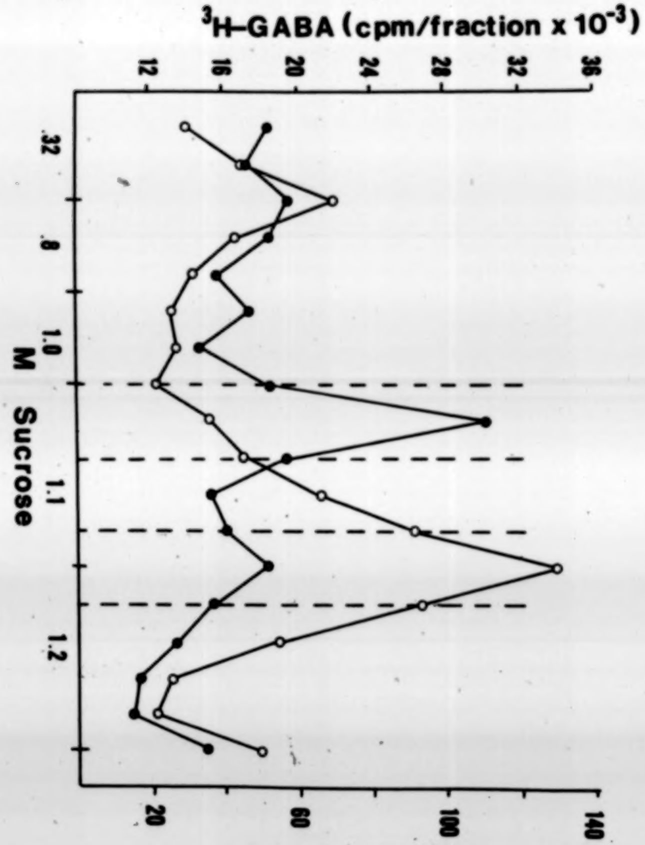
In conclusion, the evidence in favor of exocytosis seems to outweigh the evidence against this model. Experimental approaches to prove the exocytotic model is correct have been presented (Bloom, Iversen and Schmitt, 1971). In the next few years the problem of whether or not exocytosis is the correct model will probably be solved.

One aspect of catecholamine release in the CNS that is commonly overlooked in most reviews of this subject is the relationship between catecholamine release and cholinergic innervation. Phillipu (1970) has demonstrated in hypothalamic perfusion studies that atropine alone or in combination with ACh enhances the release of $^{14}$C-NE. Similarly, Hitzemann *et al.* (1971) have shown that scopolamine enhances the release of $^{14}$C-NE and $^{14}$C-DA formed from $^{14}$C-tyrosine in the whole mouse brain. The transient stimulatory effect of scopolamine on mouse motor activity may be explained by the drug's effect on adrenergic neurons. Unlike benztropine (Coyle and Snyder, 1971), scopolamine and atropine are not known to inhibit catecholamine uptake. The relationship between ACh and catecholamine release may be pertinent to our study of *d*-amphetamine, since it is known that *d*-amphetamine enhances ACh release (Deffinu *et al.*, 1970).

## 2.11 CATECHOLAMINE REUPTAKE.

In the past few years it has become generally recognized that each neurotransmitter is associated with a specific presynaptic uptake mechanism (Iversen, 1967, 1970). This specificity of uptake makes it possible to study neurotransmitter transport in rather crude brain tissue preparations. Coyle and Snyder (1969), Snyder and Coyle (1969) and Iversen and Snyder (1968) have demonstrated that the post 1000 x g supernatant of a 10% 0.32 M sucrose brain homogenate can be used to measure catecholamine, 5-HT and GABA uptake. The uptake occurs primarily into NEP which contain the respective amine (Iversen and Snyder, 1968; Kuhar *et al.*, 1970). Kuhar *et al.* (1970) have made use of the specific uptake mechanisms for NE and GABA to separate different populations of NEP on a continuous sucrose gradient. Recently in this laboratory we have modified the procedures of Kuhar *et al.* (1970) to enable us to produce bulk preparations of GABA and NE concentrating NEP (see Fig. 7). Obviously, this procedure does not produce entirely pure GABA or NE NEP; NEP which concentrate other neurotransmitters

Fig. 7. Separation of different populations of NEP on a discontinuous sucrose gradient. A crude mitochondrial fraction was suspended in Krebs-Ringer-Bicarbonate (KRB) buffer and then incubated at 37° C for 5 min after the addition of $10^{-7}$ M each $^3$H-GABA and $^{14}$C-NE. The reaction mixture was then centrifuged (10 min x 48,000 g), washed twice and finally resuspended in 0.32 M sucrose. The suspension was then layered on the discontinuous gradient indicated in the figure. The gradient was centrifuged for 90 min x 53,000 g. Two ml fractions were removed from the gradient and counted for total $^{14}$C and $^3$H.


$^{3}$H–GABA (cpm/fraction x 10$^{-3}$)
12
16
20
24
28
32
36
.32
.8
1.0
1.1
1.2
M Sucrose
20
60
100
140
$^{14}$C–NE (cpm/fraction x 10$^{-3}$)

contaminate both preparations. However, the methodology employed illustrates the usefulness of knowing that specific uptake mechanisms exist.

Like most transport systems, the neurotransmitter systems can accept compounds structurally similar to the preferred substrate. Thus, for example, DA can be taken up into NE neurons and vice versa (Snyder and Coyle, 1969). Snyder and Coyle (1969) have suggested that DA and NE transport systems can be identified by a means other than gross anatomical location. DA specific transport systems should be non-stereospecific; NE transport systems should show stereospecificity. In the caudate nucleus, *d*- and ℓ-amphetamine are nearly equipotent in blocking DA or NE uptake. However, in the cortex or other regions that contain primarily "noradrenergic" nerve endings, the *d*-amphetamine isomer is more potent to block catecholamine uptake than the ℓ-isomer. Desipramine (DMI) specifically inhibits catecholamine uptake into NE nerve endings, but not DA nerve endings. Recently, these findings of Snyder's group on the differences between DA and NE transport systems have been questioned (Ferris *et al.*, 1972). It would appear that additional experiments are necessary to confirm or refute the non-stereospecific nature of the DA transport system.

NE uptake in peripheral and central adrenergic neurons is thought to be mediated via an active transport process. Some reports suggest that NE may be accumulated against as much as a 6000-fold concentration gradient in the region of the nerve ending (Iversen, 1970). Evidence that NE is transported actively may be summarized (Iversen, 1970): (a) NE uptake is temperature dependent; (b) NE uptake is primarily $Na^+$ dependent but also requires a small amount of $K^+$ but not $Ca^{++}$ ions;

(c) Michaelis-Menton kinetics can be used to describe NE uptake; (d) NE uptake is inhibited by ouabain and metabolic poisons, e.g., CN. Bogdanski and co-workers (Bogdanski *et al.*, 1970; Tissari and Bodganski, 1971; Bogdanski and Brodie, 1969) have proposed a model of NE uptake in peripheral tissues and the CNS based on the similarities between NE uptake and that of amino acids or sugars in other tissues (Crane, 1965). Briefly, this model suggests that $Na^+$ and NE bind to the carrier on the external surface of the membrane in a cooperative manner. The $Na^+$-NE-carrier complex is then translocated through the membrane with the force for translocation being supplied by the downhill $Na^+$ gradient. On the internal membrane surface $Na^+$ and NE are released and $Na^+$ is pumped to the outside via a mechanism which is intimately tied to the $Na^+$ and $K^+$ dependent ATPase. When the $Na^+$ gradient is lowered either by ouabain or by reducing the external $Na^+$ concentration, the rate of NE uptake decreases. Recently, White and Keen (1970) have suggested that $Na^+$ and NE do not bind cooperatively to the carrier. Rather, NE binding occurs independently of the $NA^+$ concentration. Thus, while Bogdanski found that lowering the $Na^+$ concentration caused a competitive inhibition of uptake, White and Keen (1970) observed a non-competitive inhibition.

## 2.12 METHODS TO ESTIMATE DYNAMIC CHANGES IN CATECHOLAMINE FUNCTION.

With the development of fluorometric procedures sensitive enough to measure the μg quantities of biogenic amines in the CNS (see Udenfriend, 1962), a great number of neuropharmacologists and neurochemists turned their attention to examining the effects of various drugs and other stimuli on these substances. These studies have shown, however, that only a few drugs, e.g., reserpine and high doses of *d*-amphetamine, were capable of altering endogenous neuroamine levels. In all likelihood, the intense interest and popularity that the study of catecholamines and serotonin enjoys today would not exist if it were not for the development of $^3$H-NE, $^3$H-DA and $^3$H-5-HT of a high specific activity (> 1 Ci/mmol). A whole new conceptual approach to the examination of NE function was developed from the observations of Whitby *et al.* (1961) that a tracer dose of $^3$H-NE can be used to label endogenous NE stores in adrenergic neurons. It is important to remember that the usefulness of the tracer labeling approach depends on the existence of the specific uptake mechanisms (Hertting and Axelrod, 1961; Iversen, 1970) which will concentrate the $^3$H-amine in the appropriate neuron. The remainder of this review will be primarily concerned with the examination of dynamic changes in the catecholamines but it should be realized that in most cases similar results and interpretations have been obtained for 5-HT.

In 1965 Glowinski *et al.* observed that when $^3$H-NE was introduced intraventricularly into the rat brain, the $^3$H-NE disappeared in a multi-exponential fashion. Three phases of disappearance were described and each phase was thought to correspond to a different intraneuronal pool. The three pools had half-life ($t_{1/2}$) values of 70 min, 3 hr and 17 hr. These data taken in conjunction with previous studies in peripheral adrenergic tissues suggested that NE did not exist in a static single pool, but rather was "turning over" from several pools. The multi-compartmental model of the NE neuron was originally devised to explain various drug effects in peripheral adrenergic neurons. Potter *et al.* (1962) demonstrated that tyramine could release only a portion of heart NE. Tachyphylaxis to the depletion of heart NE and to the sympathomimetic response was found to occur with repeated tyramine injections. Subsequent studies (Harrison *et al.*, 1963; Iversen and Whitby, 1963; Kopin and Gordon, 1962, 1963) led credence to the proposal by Potter and Axelrod (1963) that NE exists in at least two pools within the neuron. One pool was susceptible to tyramine and other indirectly acting sympathomimetics and the other pool was susceptible to nerve stimulation.

*In vivo* and *in vitro* studies have suggested that nerve stimulation enhances NE turnover and synthesis (Oliverio and Stjarne, 1965; Gordon *et al.*, 1966; Roth *et al.*, 1966; Sedvall *et al.*, 1967; Weiner and Rabadjija, 1968; Sedvall *et al.*, 1968; Harris and Roth, 1971; Weiner *et al.*, 1972). Sedvall *et al.* (1968) have proposed that nerve stimulation preferentially releases newly synthesized NE from a small mobile pool. Glowinski (1970) has adopted this viewpoint in his extensive studies of drug effects including *d*-amphetamine on brain catecholamines. The multicompartmental model of Sedvall *et al.* (1968) probably represents the zenith of this approach. In the last five years no real new interpretation of the multicompartmental model has been forthcoming. The data which led Sedvall *et al.* (1968) and Kopin *et al.*, (1968b) to postulate that nerve impulses preferentially release newly synthesized catecholamines may be summarized.

1. After labeling the spleen with $^{14}$C-tyrosine, the specific activity of the NE released by stimulation of the afferent nerves is greater than the specific activity of NE stored in the spleen.

2. When the spleen is prelabeled (24 hr) with $^{3}$H-NE, the specific activity of the NE released by nerve stimulation is equal to that of the spleen; with continued nerve stimulation the specific activity of

XUM

the released material is less than that of the spleen.

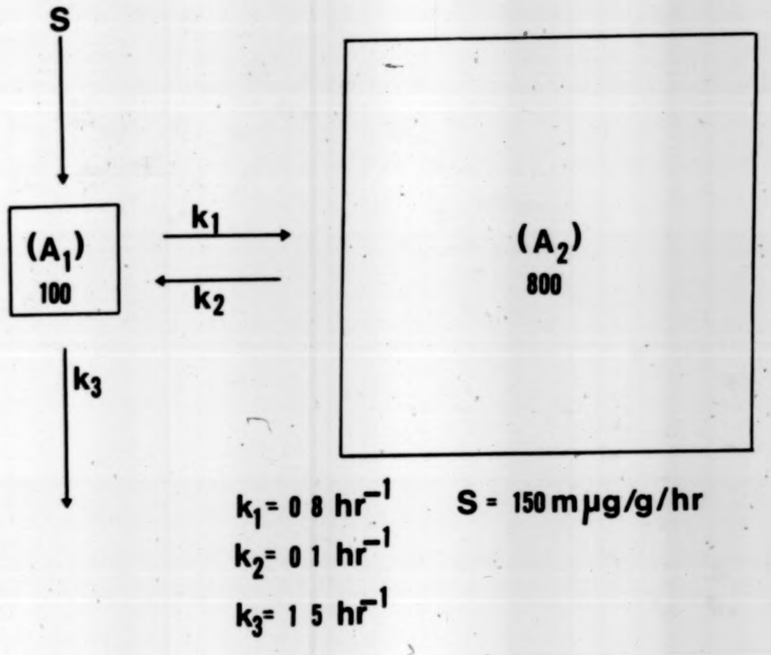
3. After labeling the spleen with $^{14}$C-tyrosine and then infusing α-methyltyrosine (α-MT), the specific activity of the NE released by nerve stimulation is similar in control and in α-MT treated preparations; the specific activity of the released NE is greater than that in the spleen.

When the time after α-MT infusion is lengthened such that the inhibition of tyrosine hydroxylase activity is near maximal, a different phenomena is observed; the release of NE after nerve stimulation is reduced but the specific activity of the released material is similar to the specific activity of the NE in the spleen. Costa (1970) has suggested that these data do not equivocally prove the preferential release of newly synthesized NE. However, his arguments also seem to be of dubious value. He stresses that it is difficult to know if the experimental design adopted by Kopin and his co-workers accurately mirrors physiological conditions. This kind of argument is circular and falls into the "why" and "why not" type of logic.

Costa, who was attempting to defend his single compartmental model, does, however, have convincing arguments in other areas.

1. The mathematical model of Sedvall *et al.* (1968) (see Fig. 8) was adopted to explain the preferential release of newly synthesized NE and to explain that it is possible for two compartments to exist even though usual probing procedures may indicate a single open compartment (see Neff *et al.*, 1968, and Costa *et al.*, 1966). Costa has shown that this model has built-in discrepancies. For example, when one attempts to calculate the value of $k_3$ from known experimental data (Westfall and Osada, 1969; Neff *et al.*, 1969, 1971), a $k_3$ value is

Fig. 8. Kinetic model of catecholamine pools as proposed by Sedvall *et al.* (1968). The relative size of the two pools is indicated by the numbers 800 and 100. $K_3$ = the rate of NE synthesis. It is important to note that $K_3$ is significantly higher than values reported elsewhere (Westfall and Osada, 1969; Neff *et al.*, 1969).


S
(A₁)
100
k1
k2
k3
(A₂)
800
k1 = 0 8 hr⁻¹
k2 = 0 1 hr⁻¹
k3 = 1 5 hr⁻¹
S = 150 mμg/g/hr

obtained which is considerably lower than that reported by Sedvall *et al.* (1969). Several other discrepancies are also discussed by Costa to which the reader is referred.

2. There are several problems encountered when using the multiexponential decline of $^3$H-NE injected intraventricularly as an argument in favor of multicompartmentation: (a) A significant portion of intraventricularly administered NE is taken up into the surrounding ependymal cells which normally do not store NE (Fuxe and Ugerstedt, 1966). (b) A significant portion of the intraventricularly administered NE will be taken up by dopaminergic neurons (see Snyder and Coyle, 1969; Fuxe and Ugerstedt, 1966). DA neurons will actively concentrate NE against a gradient unlike the ependymal cells and will probably also actively release the NE. Uptake into DA neurons can then only "muddy" any interpretation of compartmentation in noradrenergic neurons.

3. Evidence for multicompartmentation in peripheral tissues depends largely on the phenomena of tyramine tachyphylaxis in regard to NE depletion. However, Neff *et al.* (1965) have found that under appropriate conditions tyramine can deplete over 90 percent of heart NE. Neff *et al.* (1965) concluded that the tyramine resistant pool is an artifact of a rapid disappearance of tyramine perhaps caused by intraneuronal MAO.

4. A recent report by Costa's group (1974) indicates that α-MT not only blocks catecholamine synthesis, but also indirectly displaces endogenous NE stores via the formation of PONE from α-MT. Although not demonstrated by these authors, it is possible that the PONE formed will inhibit catecholamine synthesis as discussed by Kopin *et al.* (1968). Thus, the data presented by Kopin *et al.* (1968b) on the effect of α-MT

on the release of newly synthesized NE from the spleen is subject to re-evaluation.

The alternative to the multicompartmental model is the single open compartment model (Costa, 1970; Neff *et al.*, 1971). This model was developed by Costa and co-workers for the following reasons:

1. Catecholamine tissue levels are relatively stable in the face of changing rates of neuronal depolarization and of transmitter release. Superficially, these data would suggest that the rate of catecholamine synthesis equals the rate of catecholamine utilization (the steady-state condition.

2. Nerve endings are composed of several distinct morphological components, each of which play an important role in catecholamine synthesis, release, reuptake and metabolism. Ideally, the role of each of these components would be expressed in terms of individual kinetic parameters which influence synthesis and turnover. Practically, this is impossible and, as a consequence, it is necessary to approach the problem of how to express the steady-state condition with a provisionally simpler model.

3. As previously discussed, the evidence for a multicompartmental model is no better than that for a single compartmental model.

4. The simplest possible model of the adrenergic neuron is the view that catecholamines are stored in an open single compartment when newly synthesized molecules rapidly mix with the molecules stored in the compartment. In essence, the nerve ending is pictured as a black box, where tyrosine is converted to DA or NE at a rate equal to the utilization of catecholamine from the black box. Realizing that tyrosine hydroxylase is the rate limiting enzyme in the biosynthetic

sequence, it is possible to kinetically picture the conversion of tyrosine to NE occurring as a single rather than multiple step; turnover, the composite of release, reuptake and metabolism, can also be pictured as a single process. When a pulse label of tyrosine is administered, the fractional rate constant of tyrosine conversion to catecholamine can be approximated as

$$k = \frac{\dfrac{S.A.CA_{t_2} - S.A.CA_{t_1}}{t_2 - t_1}}{\dfrac{(S.A.TYR_{t_1} - S.A.CA_{t_1}) + (S.A.TYR - S.A.CA_{t_2})}{2}} \qquad (1)$$

The development of this equation and more exacting methods to determine k are given by Neff *et al.* (1971).

5. When a pulse label of $^3$H-tyrosine is administered, labeled catecholamines accumulate at a rate greater than the turnover rate (Costa, 1970). If newly synthesized catecholamines are preferentially released (Sedvall *et al.*, 1968), then one would predict that the initial rate of accumulation would be less than the turnover rate; actually, it appears as if the newly synthesized amines are preferentially retained and are released after uniform mixing. This analysis further supports the conclusion of an open single compartment.

The method developed by Costa and co-workers to determine $k_{NE}$ (or $k_{DA}$) from a pulse label of $^3$H-tyrosine agrees with other methods for determining k, e.g., the disappearance of a pulse label of $^3$H-NE and the disappearance of endogenous NE after the administration of α-MT (Glowinski *et al.*, 1965; Brodie *et al.*, 1966). However, in order to apply this method to pharmacological studies, certain conditions must be satisfied.

1. During the experimental period the endogenous levels of the precursor and product must be in a steady-state. Unfortunately, as in the present study, this condition cannot always be met. Acute *d*-amphetamine treatment causes a transient rise in brain DA; chronic PONE pretreatment deceases brain NE levels and this decrease is further enhanced by small doses of *d*-amphetamine (see Chapter 5).

2. While theoretically equation 1 can be applied at most times prior and after the time of peak product specific activity, the method is most accurate during the period when catecholamine specific activity is decreasing (see Neff *et al.*, 1971, and Costa, 1970). The half-life for the disappearance of $^3$H-NE formed from $^3$H-tyrosine is on the order of 2 to 4 hours. Ideally, the experimental period to detect drug-induced changes in $^3$H-NE turnover would last at least two or three half-life periods. Practically, this condition cannot be met in most acute drug studies; generally, much shorter time courses of 1 to 2 hours are used and, as a consequence, the factor of error is greatly increased.

After comparing the multi and single compartment models, it would not be surprising to find the investigator wishing to examine drug-induced changes in the brain catecholamines in a state of utter confusion on how to develop a proper experimental design. It does seem, though, that certain minimal guidelines have developed which are independent of the model adopted.

1. Extrapolation of drug-induced changes in the disappearance of a pulse label of $^3$H-NE (or DA) to statements concerning the rate of brain catecholamine synthesis may be misleading. Turnover will not equate with synthesis if the steady-state is disturbed. Furthermore,

it may be difficult to obtain a distribution of the $^3$H-catecholamine which is similar to the distribution of the endogenous amine. Thus, if one is interested in synthesis rates, it would seem advisable to use either a continuous infusion or a pulse of labeled tyrosine. Ideally, for the pulse labeling studies the labeled tyrosine should be administered peripherally in order to obtain a normal distribution of the precursor. However, this is not always possible because of financial considerations. For example, in my experience it is necessary to inject 100 μCi of $^3$H-tyrosine (specific activity 13 Ci/mμmol) or more per rat i.p. in order to obtain maximal cpm of 100 to 200 above background for DA and NE. Discrete brain studies or studies designed to measure metabolite formation will require more label. Additionally, it should be remembered that certain drugs, e.g., morphine (Loh *et al.*, 1973), will alter the uptake of label to the brain. The obvious alternative to the peripheral injection is to administer tyrosine via a central route, i.e., intracranially, intraventricularly or intracisternally. The intraventricular route will tend to label the dopamine nerve endings of the caudate nucleus as well as some of the norepinephrine nerve endings of the hypothalamus, thalamus and surrounding structures. The intracisternal route will label primarily the nerve ending and cell bodies of the brain stem with some labeling (approximately 20 to 30 percent of the total) occurring in the midbrain, thalamus and hypothalamus (preliminary observation). Less than one percent of the total injection reaches the neostriatum when using the intracisternal technique. Thus, this method can be used for the study of only norepinephrine synthesis, metabolism and turnover. Needless to say, the injection volume and the time interval from injection will influence the degree to which the label distributes from the site of injection. Finally, by using very small injection volumes, 1 μl or less, it is possible to specifically label discrete brain regions. In conclusion, the choice of isotope to be used and the route of injection chosen must be carefully considered. Each has its advantages and disadvantages which must be carefully weighed against each other before beginning the experiment.

2. When using a pulse label of tyrosine to estimate catecholamine synthesis, it seems appropriate to examine at least two time points when the specific activities of NE and DA are increasing. If, as Costa (1970) suggests, there is a mixing of the newly synthesized amines with the stores that must occur prior to utilization, then one might conclude that time points chosen soon after injection will primarily measure synthesis. Unfortunately, at these early times/there is the additional factor of precursor distribution. There is likely to be some lag between the time of injection and the time when the tyrosine is maximally distributed to the sites of synthesis. Thus, it is important to choose for analysis only those time points on the incorporation curve where NE or DA specific activity are increasing at a constant rate. Since the initial phase of incorporation is probably a nonequilibrium condition, data obtained from this phase are probably best suited for only comparative purposes. A useful calculation for analyzing data during this time is the conversion (Azmitta *et al.*, 1969) or accumulation (Loh *et al.*, 1973) index. The CI or AI may be written as

$$\text{CI(AI)} = \text{m}\mu\text{mol CA} = \frac{\text{dpm CA}}{\text{S.A.Tyr.}} \qquad (2)$$

By using equation 2, one can calculate the amount of catecholamine which was formed and has remained in the store at any time after the pulse label. In our opinion, the term conversion index was somewhat misleading and, consequently, ~~we~~ renamed is the accumulation index.

3. The discussion to this point has dealt primarily with *in vivo* models. However, the *in vitro* model, when applicable, is a particularly useful technique. The methodology described by Harris and Roth (1971) for determining catecholamine synthesis rates in brain slices is a good example of *in vitro* methodology. The advantages of using a closed system where one can determine and not speculate or infer on the rates of synthesis, utilization, release and metabolism is obvious. The data obtained by Harris and Roth (1971) are remarkably similar to *in vivo* data and suggest that when *in vitro* techniques are carefully applied to problems relating to catecholamines, the results may indeed reflect the *in vivo* situation.

## 2.13 *d*-AMPHETAMINE - INTRODUCTION.

To say that amphetamine is a favorite tool of pharmacologists would be an understatement. The literature concerning the effects of amphetamine is voluminous; the areas of interest that have been investigated in detail vary from conditioned avoidance responses to free-fatty acid mobilization or from the use of amphetamine to induce psychoses to the treatment of hyperkinetic children. For our purposes, though, it is necessary to briefly review the literature in only four areas. These are (a) amphetamine metabolism, (b) the effect of amphetamine on brain catecholamines, (c) the effect of amphetamine on locomotor and stereotyped behaviors, and (d) the effects of chronic amphetamine intoxication on the brain catecholamines and behavior.

## 2.14 AMPHETAMINE METABOLISM.

Reviews and articles on the adsorption, distribution and excretion of *d*-amphetamine have appeared (Alleva, 1963; Asatoor *et al.*, 1965; Beckett and Rowland, 1965; Consolo *et al.*, 1967; Gunne, 1967; Wilkinson and Beckett, 1968; Beckett and Brookes, 1970; Smith and Dring, 1970; Vree and von Rossum, 1970). For our purposes, though, it is the nature of amphetamine metabolism which is of interest, since one of the amphetamine metabolites, p-hydroxynorephedrine (PONE), is a noradrenergic false transmitter. The two major pathways of amphetamine metabolism are illustrated in Fig. 9 and 10. The oxidative deamination (Fig. 9) of amphetamine was first studied in detail by Axelrod (1955). Subsequent investigations (see ref. in Dring *et al.*, 1968) have completed the picture. Amphetamine is first deaminated by an unknown mechanism to form phenylacetone and ammonia. Phenylacetone may undergo a number of different transformations. It may be reduced to the secondary alcohol, benzmethylcarbinol, followed by glucuronide conjugation. A minor pathway involves the formation of the O-sulfate conjugate of the enolic form of phenylacetone. The major fate of phenylacetone, however, is further oxidation to benzoic acid which is excreted as hippuric acid. Like the first oxidative step, this mechanism of this second oxidation is presently not known. With the exception of the rat, oxidative deamination is a major metabolic pathway in all species studied (Ellison *et al.*, 1966; Smith and Dring, 1970) as evidenced by the formation of benzoic acid. In general, benzoic acid accounts for 20 to 35 percent of the injected dose, although in the guinea pig benzoic acid accounts for over 60 percent. The rabbit has the most interesting picture of deamination with 23 percent of the injected dose appearing benzoic acid,

Fig. 9. Oxidative deamination of amphetamine from Smith and Dring, 1970. A = amphetamine; B = phenylacetone; C = benzoic acid; D = hippuric acid; E = benzylmethylcarbinol; F = benzylmethylglucuronide; G = phenylacetone sulfate of enolic.

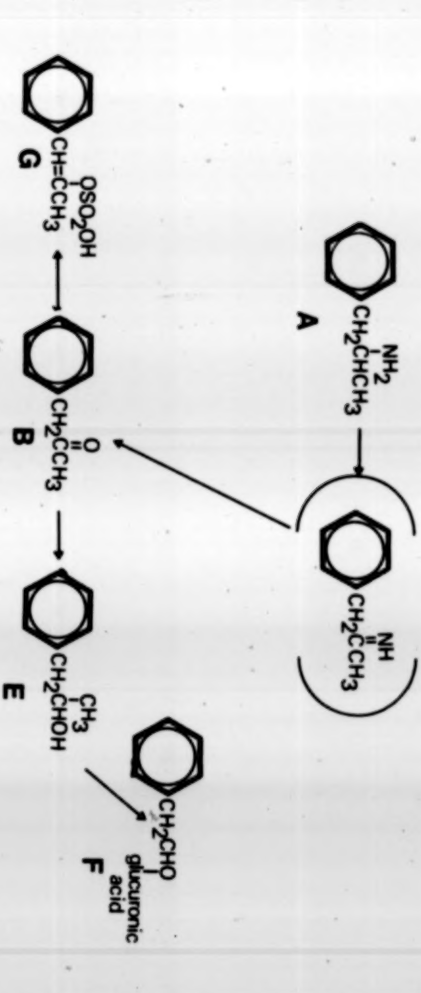
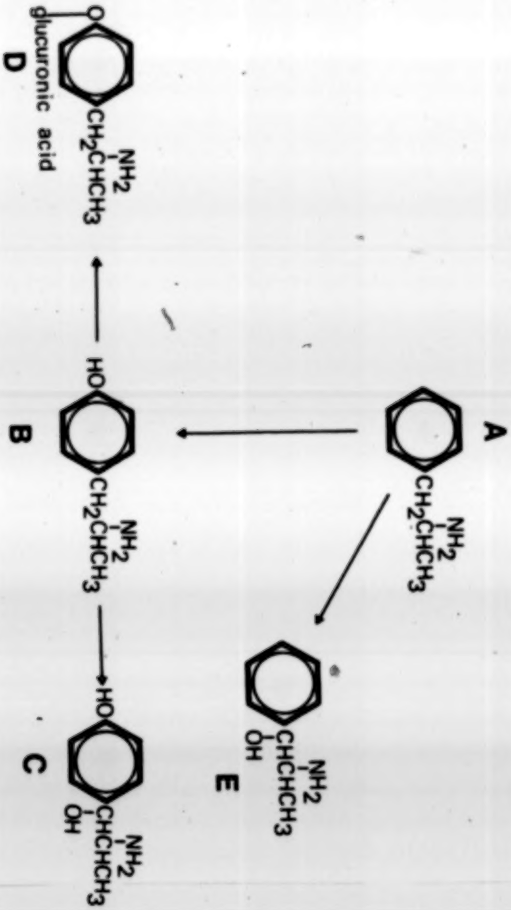

A
B
C
D
E
F
G
glucuronic acid

Fig. 10. Hydroxylation reactions in amphetamine metabolism from Smith and Dring, 1970. A = amphetamine; B = *p*-hydroxyamphetamine; C - *p*-hydroxynorephedrine; D = glucuronide of *p*-hydroxyamphetamine; E = norephedrine.


A
B
C
D
E
glucuronic acid
HO
OH
O
$NH_2$
$CH_2CHCH_3$
CHCHCH$_3$

8 percent as the benzymethylcarbinol conjugate and 22 percent as the phenylacetone conjugate. Axelrod (1955) has suggested that the hepatic microsomal system responsible for deamination is inhibited to various degrees by a heat labile factor in the rat, dog and guinea pig.

To a major degree in the rat and to a minor degree in other species (see Table 4), amphetamine is hydroxylated in the para position (see Fig. 10). There is no stereospecificity in this reaction and, in fact, some evidence suggests the ℓ-isomer is more easily metabolized than the ℓ-isomer (Dring, Smith and Williams, unpublished observation in Smith and Dring, 1970). However, unlike ℓ-p-hydroxyamphetamine, *d*-p-hydroxyamphetamine can undergo an additional reaction catalyzed by DA-β-hydroxylase to form *d*-p-hydroxynorephedrine (PONE) (Goldstein and Anagnoste, 1965). PONE is a noradrenergic false transmitter (Kopin *et al.*, 1965). The accumulation of PONE in noradrenergic nerve terminals has been evoked as the mechanism by which single or repeated doses of *d*-amphetamine produce the persistent depletion of NE stores in peripheral and central neurons (Brodie *et al.*, 1969, 1970; Costa and Groppetti, 1970). Brodie *et al.* (1969) have suggested that since PONE is a weak sympathomimetic, its accumulation may account for tolerance or habituation to certain *d*-amphetamine effects. Since PONE can be released by electrical stimulation (Fischer *et al.*, 1965), nerve activity (Gill *et al.*, 1967), and by successive doses of *d*-amphetamine (Brodie *et al.*, 1970; Costa and Groppetti, 1970), Costa and Groppetti (1970) have suggested that "... if p-hydroxynorephedrine is present in brain tissue of amphetamine abusers, it may be important in producing their depression and psychotic reactions". Experimental evidence indicating that the decreased pressor response to tyramine in man seen after

## TABLE 4

### Species Variation in the Aromatic Hydroxylation of Amphetamine to p-OH-Amphetamine

| Species | Percent excreted in urine in p-hydroxylated metabolite(s) [a] |
|---|---|
| Man | 3 |
| Rhesus and squirrel)<br>monkey, greyhound ) | 2-6 |
| Dog | 20 |
| Mouse | 14 |
| Rat | 60 |
| Rabbit | 6 |
| Guinea pig | 0 |

[a] Data from Smith and Dring, 1970; Axelrod, 1953, 1954; Bralet *et al.*, 1968; Walkenstein *et al.*, 1955; Ellison *et al.*, 1966.

chronic *d*-amphetamine administration is related to the accumulation of PONE has been presented by Cavanaugh *et al.* (1970). Recently, Taylor and Sulser (1973) have reported on the acute effect of intraventricularly administered PONE on motor activity and the disposition of a pulse label of $^3$H-NE. PONE was found to deplete brain NE, increase the formation of $^3$H-normetanephrine and decrease the formation of $^3$H-deaminated catechol metabolites. Behaviorally, PONE was found to produce stereotyped behavior. Although these studies demonstrate an amphetamine-like effect of PONE, the data are suspect for at least two reasons.

1. The dose of PONE was extremely large (40 μg). Since PONE is commercially available only as a racemic mixture of the four isomers, the observed chemical and behavioral effects may be caused by any one of the isomers when such a large dose is administered.

2. As previously discussed, intraventricular injections are best suited to study DA and not NE metabolism. The observation that PONE primarily caused stereotyped behavior probably indicates that a substantial portion of the injected PONE was taken up by the dopaminergic nerve terminals of the neostriatum and caused the displacement of DA.

The influence of false transmitters on NE synthesis has been carefully studied by Kopin *et al.* (1968) in the rat heart. These authors have observed that false transmitters had two effects on NE synthesis. (1) By displacing endogenous NE stores, false transmitters inhibit the formation of $^{14}$C-catecholamine from $^{14}$C-tyrosine presumably by a feedback control mechanism. (2) False transmitters also inhibit the formation of $^3$H-NE when $^3$H-DOPA is the substrate. The mechanism thought to be involved here is one whereby the false transmitters, stored in the vesicle, prevent the access of the newly synthesized DA to the dopamine-β-hydroxylase. The inhibition of catecholamine synthesis at the level of tyrosine hydroxylase is a fairly transient effect, disappearing as the displaced NE is metabolized by MAO. The block at the level of DA-β-hydroxylase is a more persistent effect in that it is related to the turnover of the stored false transmitter. As would be expected, the phenylisopropylamine false transmitters have a more persistent effect than the phenylethylamine false transmitters, since the former group is not a substrate for MAO.

## 2.15 AMPHETAMINE AND BRAIN CATECHOLAMINES.

Stein (1964) has presented an excellent review of the proposed central mechanisms of *d*-amphetamine action as related to the brain biogenic amines for the years prior to 1964. Four main theories were offered. (1) Amphetamine inhibits MAO activity directly and thus increases the effective amount of neurotransmitter at the synapse. (2) Vane (1960) and Gelder and Vane (1962) suggested that amphetamine directly stimulates brain tryptamine receptors. (3) Smith (1963, 1965) proposed that amphetamine directly stimulates brain catecholamine receptors. Proposals 2 and 3 were put forth to explain the fact that reserpine, which depletes brain biogenic amines, does not diminish the excitatory actions of amphetamine (Smith, 1963; Rech, 1964) and because Vane (1960) had demonstrated in an isolated gut preparation that amphetamine has tryptamine receptor stimulating properties.

McLean and McCartney (1961) and Moore and Lariviere (1963) demonstrated that fairly large doses of *d*-amphetamine significantly reduced the levels of brain NE but had little effect on brain 5-HT or DA. Moore (1963) further showed that the relative ability of the *d*- and *l*-amphetamine isomers to deplete brain NE correlated well with their ability to produce central stimulation. These data, taken in conjunction with other studies, led Stein (1964) to propose a fourth theory on the mechanism of amphetamine action; amphetamine causes central stimulation indirectly by releasing brain NE. NE is thought to be an activating neurotransmitter in that its release results in excitement arousal, increased alertness, etc. (Poschel and Ninteman, 1963; Schildkraut *et al.*, 1967; Kety, 1967). These conclusions correlated well with the known anatomical localization of NE nerve endings in the reticular activating system and the diencephalic reward centers (Andén *et al.*, 1966). Based on physiological and psychological evidence (Kaada, 1951; Hernández-Peron, 1963; Brutkowski, 1964; Clemente and Sterman, 1967; Wise and Stein, 1970), it is likely that the mechanism by which NE facilitates behavior, at least as concerns its effect on the forebrain, is to cause inhibition of behaviorally suppressant cell groups. Thus, amphetamine may cause CNS stimulation by disinhibition.

Support for Stein's indirect mechanism of *d*-amphetamine action soon came from many laboratories. In 1965, Spector *et al.* reported that α-methyl-tyrosine (α-MT) inhibited catecholamine synthesis at the level of tyrosine hydroxylase and had no effect on brain serotonin. Subsequently, it was shown that α-MT blocked the hyperactivity and stereotyped behaviors induced by low doses of *d*-amphetamine (Weissman *et al.*, 1966; Hanson, 1967; Sulser *et al.*, 1968; Dominic and Moore, 1969). These authors demonstrated that it was not necessary for α-MT to significantly deplete the brain catecholamines in order to inhibit amphetamine's action; rather, it was necessary only to inhibit catecholamine synthesis. From these data, it was postulated that amphetamine acted primarily on a small mobile pool of newly-synthesized catecholamines. It is important to remember that at this time the multiple pool concept discussed previously was in vogue and the α-MT-amphetamine data supported this view.

Additional evidence for the indirect mechanism of action hypothesis came from Axelrod's group. In 1960 Axelrod and Tomchick showed that in mice pretreated with *d*-amphetamine, the metabolism of intravenously administered $^3$H-NE increased threefold. Amphetamine was also found to block the uptake and/or retention of $^3$H-NE in heart, spleen, adrenal gland and liver but not in skeletal muscle. To determine if amphetamine was blocking uptake and/or increasing release in peripheral tissues, Axelrod *et al.* (1962) examined the effect of amphetamine on the disposition of $^3$H-NE when the drug was given before and after the pulse label. Amphetamine caused an 80 percent reduction in $^3$H-NE accumulation when administered before the pulse and a 40 percent reduction when administered after. These data indicated that amphetamine both blocks reuptake and increases release. Similar results were subsequently obtained when the pulse of $^3$H-NE was administered intraventricularly (Glowinski and Axelrod, 1966; Glowinski and Axelrod, 1965; Glowinski *et al.*, 1966). Glowinski and Axelrod (1965) found that the reason amphetamine causes CNS stimulation and reserpine causes CNS depression, while both drugs release catecholamines, was due to the differences in metabolism. Reserpine increased the deaminated metabolites of NE and decreased the O-methylated metabolites. Amphetamine had the opposite effect. These data suggested to the authors that in the reserpinized animals NE is released and metabolized by MAO intraneuronally. In contrast, amphetamine released NE in a physiologically active form, the drug prevented NE uptake and, consequently, NE was metabolized by the extraneuronal COMT. A more detailed investigation of the mechanism by which amphetamine decreases oxidative NE metabolism and increases O-methylation has been presented by Rutledge (1970).

Overall, the effect of α-MT on amphetamine-induced stimulation and the effect of amphetamine on the disposition of $^3$H-NE led investigators to propose that the mechanism of amphetamine action was to increase the release and block the reuptake of newly synthesized catecholamines. Based on the studies of Randrup and Scheel-Krüger (1966), it was concluded that the amphetamine effect on NE resulted in increased locomotion while higher doses of amphetamine affected the DA stores and caused stereotyped behavior. As with increased locomotor activity, α-MT but not reserpine blocks the amphetamine induced increase in stereotyped behavior (Randrup and Munkvad, 1970).

Arguments against the proposed mechanism of *d*-amphetamine action stated in the previous paragraph have been often voiced and have occasionally appeared in print (Costa and Groppetti, 1970). These arguments and the usual rebuttal may be summarized.

1. α-MT produces severe behavioral depression in the doses generally employed; the fact that α-MT blocks the amphetamine induced stimulation may simply be the result of this general depressant action. Secondly, one cannot dismiss the possibility that in large doses α-MT will act as a receptor blocker. In rebuttal, Weissman *et al.* (1966) have shown that low doses of α-MT (50 mg/kg) which do not produce significant behavioral depression and only a transient decrease in brain catecholamine levels, will inhibit amphetamine's actions but not that of a number of other CNS stimulants, e.g., pentylenetetrazol or strych-

nine whose presumed mechanism(s) of action are unrelated to the brain catecholamines. The behavioral depression caused by α-MT is not correlated with the brain levels of α-MT but rather with the depletion of brain DA and NE (Moore, 1966; Rech *et al.*, 1966). These findings would argue against the hypothesis that α-MT is a receptor blocker. α-MT has only a limited ability to block amphetamine effects. For example, Brodie *et al.* (1969) have found that while α-MT can block the effects of a single dose (3 mg/kg, i.p.) of *d*-amphetamine in the rat, when a second dose is given one half hour later, stimulation results. Similarly, it was found in mice that α-MT affords protection against amphetamine only when the dose of amphetamine is 4 to 5 mg/kg or less (Hitzemann, 1970). When higher doses of amphetamine were used, no amount of α-MT would block the stimulatory response. However, the stimulation caused by a dose of 10 mg/kg of *d*-amphetamine was blocked by α-MT plus reserpine. When still higher doses of *d*-amphetamine were used (e.g., 20 mg/kg), the combination of α-MT plus reserpine was ineffective. Somewhat similar results to these just described have been reported by Smith *et al.* (1973) and Rech and Stolk (1970). Overall, these data suggest that low doses of amphetamine depend on the uninterrupted synthesis of catecholamines; when higher doses of amphetamine that can deplete catecholamine stores are used, it is necessary to both block synthesis and decrease endogenous catecholamine levels in order to block the amphetamine effects; when very large doses of amphetamine are used, it is likely that amphetamine is either acting as a direct receptor stimulant or that it is affecting the metabolism of some other putative transmitter, e.g., ACh.

2. The fact that reserpine pretreatment alone does not block

amphetamine-induced stimulation was disregarded as an important argument against the indirect mechanism of action when it was realized that reserpine does not interfere with catecholamine synthesis (Andén *et al.*, 1964). Presumably, in the reserpinized animal the newly synthesized amines are metabolized by MAO intraneuronally. After amphetamine administration the newly synthesized amines are released in such a way that they are protected from MAO. However, there are certain lines of evidence which argue against this view. (a) Rutledge and Weiner (1967) have shown that in the heart reserpine prevents the access of DA to the DA-β-hydroxylase. Thus, while reserpine may not depress catechol synthesis, NE synthesis is depressed. Although this phenomenon has not yet been demonstrated in brain tissue, if we assume that it occurs there as well it is difficult to reconcile these data with the known requirement for amphetamine of a functional pool of NE and the fact that reserpine does not block and may even potentiate amphetamine's effects. It should be noted, however, that some investigators, notably Costa's group, have suggested that brain DA and not brain NE plays the more important role in the amphetamine-induced increase in locomotor activity. This theory is reinforced by the observations of Randrup and Munkvad (1970) that much of the amphetamine-induced increase in locomotor activity consists of stereotyped patterns and is not exploratory behavior per se. (b) The preponderance of chemical evidence (Section 2.11) indicates that catecholamine release occurs via exocytosis. Clearly, exocytosis will be blocked in reserpinized animals and, thus, one wonders by what mechanism amphetamine increases release. One must either conclude that release from a small functional pool is not dependent on the vesicle or that amphetamine causes stimulation in reserpinized animals by a direct mechanism. (c) Additional data which favors the view that in reserpinized animals amphetamine is still releasing newly synthesized catecholamines comes from the observation that α-MT blocks the behavioral effects of amphetamine in reserpinized animals (Randrup and Munkvad, 1970).

3. The inhibitory effect of α-MT on amphetamine-induced stimulation almost by necessity evokes an explanation involving the multiple pool concept of the adrenergic nerve terminal; the experimental evidence that multiple pools exist is, however, quite controversial and would seem to be a poor foundation upon which to build a proposed mechanism of drug action. Wise and Stein (1970) have suggested that the *de novo* synthesis of catecholamines is not required for the behavioral facilitation caused by amphetamine. Rather, these authors have attempted to present evidence that the amphetamine's effects depend on the availability of NE in functional or physiologically active pools. Without going into detail here, I would refer the reader to Section 2.13 where the arguments for and against multiple compartmentation have been presented. Briefly stated, the multiple compartmentation hypothesis attempts to explain the preferential release of newly synthesized catecholamines by postulating the existence of a small physiologically active pool of NE at the synapse (Sedvall *et al.*, 1968). Obviously, the questions concerning the compartmentation of catecholamine stores must be resolved before the mechanism of amphetamine action can be understood.

4. The assignment of particular roles for NE and DA in the amphetamine-induced behaviors is far too simplistic a concept; a wide variety of pharmacological evidence simply does not support such a conclusion.

There can be no firm rebuttal to this argument. Evidence for and against this hypothesis is presented in the next section.

## 2.16 AMPHETAMINE, CATECHOLAMINES AND BEHAVIOR.

Stein and Seifter (1961), Stein (1964), Wise and Stein (1970) have demonstrated that amphetamine facilitates operant behavior through the release of NE and not DA. Although facilitation of operant behavior and generalized hyperactivity are different behavioral phenomenon, the possibility that the two behaviors are linked biochemically, neuroanatomically and phychologically must be considered (Hill, 1970). Along with hyperactivity, the most obvious behavior caused by amphetamine is stereotyped activities, e.g., continuous sniffing, grooming and gnawing movements. Apomorphine causes similar effects (Ernst, 1969). For quite some time it has been realized that stereotyped behaviors have a neuroanatomical locus in the corpus striatum (Amsler, 1923; Clymer and Seifter, 1947). In 1959, Bertler and Rosengren demonstrated that 70 to 80 percent of the brain DA is concentrated in the corpus striatum where it is localized in the nerve endings of the nigro-striatal nerve system (Garattini and Valzelli, 1965; Randrup and Munkvad, 1968; Matsuoka *et al.*, 1964). Pharmacological evidence incidates that amphetamine-induced increases in locomotor activity but not stereotyped behavior are inhibited by NE α or β receptor blockers (Carlsson and Lindqvist, 1964; Laverty and Sharman, 1965). The reports cited in this paragraph have been mentioned to set the groundwork for a very important short communication by Randrup and Scheel-Krüger in 1966. These authors showed that the administration of the DA-β-hydroxylase inhibitor, diethyldithiocarbamate (DDC), blocked the amphetamine-induced increase in locomotor activity but had no effect on the increase in stereotyped behaviors. This report was subsequently confirmed by Maj and Przegalinski (1967). These data, in light of previous findings, were taken as evidence that amphetamine induced hyperactivity is primarily related to activation of brain NE receptors while the increased stereotyped behaviors are primarily related to activation of brain DA receptors.

Amphetamine produces stereotyped behaviors in many and probably all mammalian species and also in birds but not in lower vertebrates, e.g., eels and lizards (Randrup and Munkvad, 1967, 1968; Nilakantan and Randrup, 1969). The stereotyped activities usually appear after high doses of amphetamine or subsequent to a period of increased locomotor and rearing activities when lower doses of *d*-amphetamine are employed (Randrup and Munkvad, 1970). The inverted "U" dose response for locomotor activity caused by amphetamine (Smith, 1963, 1965) is the result of non-locomotor stereotyped activities being the predominant behavioral response at high drug levels.

In mice and rats, stereotyped behaviors consist of highly repetitive licking, grooming and biting behaviors. In man, not entirely dissimilar behaviors are observed. Continuous chewing or teeth-grinding movements with rubbing of the tongue along the inside of the lower lip have been reported (Aschcroft *et al.*, 1965; Connell, 1966; Mattson and Calverly, 1969). More complex stereotypic behaviors are also seen in humans. These include repeating a meaningless mechanical task (Kramer *et al.*, 1967), bathing all day, repeatedly cleaning up the house, or engaging in non-ejaculatory intercourse for extended periods (Scher, 1966). More detailed descriptions of stereotyped behaviors in man and other animals are presented by Randrup and Munkvad (1967, 1970).

The hypothesis that DA serves a primary role in the production of amphetamine-induced stereotypy is supported by a great deal of pharmacological and physiological data. (1) Ampnetamine induced stereotypy can be blocked by α-MT (Randrup and Munkvad, 1966; Hanson, 1967) and the stereotypy can be restored by DOPA (Randrup and Munkvad, 1966). (2) In fairly high doses, DOPA alone can produce stereotyped behavior; this effect is not diminished by reserpine or DDC, compounds which diminish NE but not DA synthesis (Scheel-Krüger and Randrup, 1967). (3) More recent reports than those already cited showing that neuroleptics can block amphetamine-induced stereotypy have appeared (Fog *et al.*, 1968; Juorio *et al.*, 1966; Janssen and Allewijn, 1968). (4) Stereotyped behavior was observed after bilateral microinjections of DA, DOPA, apomorphine and anticholingergics into the corpus striatum (Ernst and Smelik, 1966; Fog *et al.*, 1967, 1968). (5) Lesions in the corpus striatum prevent amphetamine-induced stereotypy (Fog *et al.*, 1969).

Although DA seems to have a primary role in the production of amphetamine-induced stereotypy, other putative neurotransmitters may have important secondary roles. For example, stereotypes are not only caused by compounds with a phenylethylamine structure, but also by anticholinergics and some indoles. Thus, stereotypy may result from the interaction of several neuroamines, e.g., DA and ACh, and the ratio of receptor activities may be the critical factor which controls the production of stereotypy.

In addition to stereotypy, the most predominant behavioral pattern observed after amphetamine administration in small animals is a general increase in locomotor activity. However, if one were to carefully scrutinize the animal's total motor activity repertoire, it is likely that some components increase and others decrease in frequency (Bindra and Baran, 1959). Thus, it would probably be more accurate to describe the increase in locomotor activity as the net result of the selective facilitation and inhibition of various motor responses (Hill, 1970). I have mentioned these points only to remind the reader that locomotor activity is a complex and not a simple behavioral pattern. If one observes mice and rats (and probably other species as well) administered small doses of *d*-amphetamine, two general patterns of increased locomotor activity are seen. One pattern is increased exploratory activity (Gupta *et al.*, 1971; Gupta and Holland, 1972). Exploratory activity can be easily observed by simply removing a naive mouse or rat from its home cage and placing him in a different environment, e.g., an open-field apparatus. The animal quickly engages in a behavioral pattern which can only best be described as exploration. The locomotor activity movements appear random. Occasional rearing movements which are also observed can be considered part of the exploratory motor response (Gupta, 1971). One might suggest that amphetamine has exaggerated those controls in behavior which determine the animal's response to environmental stimuli. In a previous study (Hitzemann, 1970), various pharmacological manipulations were used to demonstrate that NE plays a primary role in the amphetamine induced increase in exploratory activity. For example, in mice administered α-MT plus DDC, DOPS but not DOPA restored the amphetamine effect.

The second type of locomotor response caused by low doses of *d*-amphetamine is stereotyped running. Lat (1965) and Schefring (unpublished observation in Randrup and Munkvad, 1970) have observed that amphetamine caused increased locomotion which was restricted to a part of the activity arena where the rats ran back and forth many times. In addition, I have observed amphetamine treated mice which will run continuously around the periphery of the activity arena and, unlike the placebo, will never transverse the middle of the arena. This behavior is remarkably similar to the "running fit" observed in morphine treated mice. The highly repetitive nature of these locomotor movements suggested to Randrup and Munkvad (1970) that the behavior may be considered a stereotyped activity. The implication of their observation is that this form of increased locomotor activity is related to activation of brain DA neurons.

Understanding that amphetamine increases different kinds of locomotor activity is important for the following reason. In recent years some controversy has arisen as to which catecholamine, DA or NE exerts the most important role in increased locomotor activity. Unfortunately, the distinction has not been made as to whether or not the various pharmacological manipulations affected exploratory or stereotyped locomotor activity.

As previously mentioned, Randrup and Scheel-Kruger (1966) suggested that *d*-amphetamine-induced locomotor stimulation results from activation of NE "receptors" in the CNS. However, some reports suggest that activation of DA "receptors" is also required for psychomotor stimulation. Fuxe and Ugerstedt (1970) have observed that increased NE "receptor" alone is not sufficient to cause increased locomotion; DA "receptors" must be activated as well. Ahlenius and Engel (1971) have suggested that activation of DA receptors is important for elementary locomotor functions while additional NE receptor stimulation is important for complex behaviors such as conditioned avoidance responses (see Wise and

Stein, 1970). Costa and Groppetti (1970) and Persson (1970) have found that doses of *d*-amphetamine which primarily increase locomotor activity increase DA but not NE turnover. In contrast to these studies which suggest an important role for brain DA in locomotion, Chan and Webster (1971) have concluded in agreement with Randrup and Munkvad (1967) that any role of DA in maintaining activity is secondary in importance to that of NE. Also, Bizzi *et al.* (1970) have found that fenfluramine, which decreases locomotor activity, increases brain DA turnover but has no effect on NE. The differences between the reports cited above may in part be related to the problems involved in measuring locomotor activity. It should also be pointed out that the methodologies employed by the various authors were generally different.

## 2.17 CHRONIC AMPHETAMINE INTOXICATION, BRAIN CATECHOLAMINES AND BEHAVIOR.

Despite the fact that chronic amphetamine intoxication has been a drug problem of some importance for over 30 years, there has been little non-clinical research in this area. Repeated injections of amphetamine have been found to produce tolerance to the amphetamine-induced facilitation of certain complex behaviors (Schuster and Zimmerman, 1961; Fischman and Schuster, 1974; Schuster *et al.*, 1966), hyperthermia (Brodie *et al.*, 1970) and anorexia (Harrison *et al.*, 1952). Reports indicating that tolerance develops to the amphetamine-induced locomotor stimulation have been both positive (Yagi, 1963) and negative (Brodie *et al.*, 1969; Schuster and Zimmerman, 1961; Lewander, 1971). Lewander (1971) has observed that repeated injections of a large dose (15 mg/kg) of *d*-amphetamine to rats does not result in tolerance or habituation to amphetamine stereotypies. Gunne and Lewander (1968) have shown that short term chronic amphetamine treatment in rats caused a gradual depletion of catecholamines in the heart and brain and an increased urinary excretion of catecholamines. With long term chronic amphetamine injections, urinary catecholamine levels began to return to normal, suggesting that some tolerance may have developed to the increased excretion of catecholamines. Javoy *et al.* (1968) have found that chronic (4 days) treatment with moderate doses of *d*-amphetamine (5 mg/kg) did not increase NE turnover in the brain stem-mesencephalon and cortex-diencephalon.

The clinical features of acute and chronic amphetamine intoxication have been well documented (see references in Connell, 1958, and Kalant, 1966). An historical perspective of amphetamine abuse and the legal-medical aspects of the problem has been discussed by Brecher (1972). Amphetamine intoxication is known to produce complex visual and olfactory hallucinations, depersonalization, hypersexuality and, most classically, paranoid reactions often developing into paranoid schizophrenia. Since individuals who abuse amphetamines are clearly not "normal", the following question is often asked. Does amphetamine actually induce a psychosis or are we merely observing the exacerbation of an existing problem? In 1958 Leake concluded that individuals who abuse amphetamines in sufficient amounts to produce psychosis were probably psychotic prior to taking the drug. While this may be the case in some individuals, more recent evidence indicates that a pre-existing psychosis is not a "requirement" for amphetamine abuse or amphetamine-induced psychosis. For example, Kramer *et al.* (1967) have observed that, in their experience, only a small percentage of the individuals abusing large amounts of amphetamine could be considered schizophrenic.

Griffith *et al.* (1970) have found that in six subjects with no history of psychosis or personality pattern disturbances the administration of *d*-amphetamine in small amounts for 5 days or to tolerance caused the development of a transitory psychosis in five individuals.

The adverse effects of acute and chronic amphetamine intoxication are not limited to the development of overt psychoses. Oswald and Thacore (1963) observed that amphetamine suppressed paradoxical or REM sleep, tolerance developed to this effect and, upon drug withdrawal, paradoxical sleep increased in duration. Readministration of the drug abolished this abstinence syndrome. Signs of dependence were evident for two weeks or more after discontinuing drug treatment. Kramer *et al.* (1967) have suggested that the exaggerated sleepiness following withdrawal of amphetamine is a sign of physical dependence. As stated by these authors, "The depth and length of the withdrawal sleep and the fatigue which lasts for several weeks afterward cannot be explained by chronic fatigue alone, but that physiological biochemical adaptations occur with chronic use of amphetamines which are analogous to those changes hypothesized to explain the abstinence syndrome due to opioids or barbiturates." The stereotypy behaviors observed after amphetamine intoxication have been previously discussed (Section 2.16). Additional clinical symptoms of amphetamine misuse, e.g., convulsions, are described in a review by Kalant (1966).

Several lines of evidence suggest that the adverse behavioral effects including psychosis caused by chronic and occasionally acute amphetamine intoxication are related to a dysfunction of the brain catecholamines. The determination that the DA of the corpus striatum is involved in amphetamine-induced stereotypes is relevant to understanding amphetamine psychosis for the following reasons: (a) the anti-psychotic potency of various neuroleptics correlates well with their ability to block stereotyped behaviors in animals (Janssen *et al.*, 1965; Janssen, 1968); (b) chlorpromazine (and presumably other neuroleptics as well) is effective in the treatment of amphetamine psychosis (Griffith 1970); (c) biochemical evidence indicates that neuroleptics are potent DA receptor blocking agents (Carlsson and Lindqvist, 1963; Laverty and Sharman, 1965); (d) some evidence suggests that psychotic disorders are at least partially related to a dysfunction of the basal ganglia (Domino *et al.*, 1968; Domino, 1968; Janssen, 1968); (e) the administration of drugs which elevate brain DA tends to exacerbate schizophrenic symptomatology (Heath *et al.*, 1965).

Weil-Malherbe (1967) has suggested that the arousal theory of schizophrenia (see Kornetsky and Mirsky, 1966) may provide a theoretical framework in which the various aspects of mental disorders can be interpreted. Clearly, there is a wealth of literature to support this viewpoint (see references in Weil-Malherbe, 1967). Ellinwood (1967) has suggested that amphetamine-psychosis may likewise result from the overstimulation of arousal systems caused indirectly by the amphetamine induced release of NE. However, Griffith *et al.* (1970) in a well controlled study noted that individuals administered repeated doses of amphetamine appeared depressed, suggesting a decrease in the arousal mechanisms had occurred.

Domino (1969) has proposed that the pharmacological model of psychosis, based largely on the effects of amphetamine, suggests that schizophrenics may have "... a multiple disturbance of neurotransmitters including too much functional dopamine or related amines, too much serotonin or related amines like tryptamine and too little acetylcholine. Inasmuch as most neurotransmitters, either in deficiency or excess, produce opposite effects to optimal levels, the problem becomes very complex from both a pathobiologic and therapeutic point of view." It is to understanding some aspects of this complex problem that the research described in this dissertation is addressed.

# CHAPTER 3

# METHODOLOGY

## 3.1 INTRODUCTION.

The somewhat diverse nature of the research described in this dissertation does not easily lend itself to a unifying description of the methods and procedures involved. In order to circumvent this problem, the details of the experimental procedures used, i.e., drug doses and schedule of injections, peculiar to the particular experiment are presented in the individual chapters. Since the chemical and behavioral methods showed considerable overlap from project to project, it is possible to generally describe the methods employed in one chapter.

## 3.2 MATERIALS.

All general chemicals were of reagent (A.C.S.) grade. Special biochemicals were obtained from the following suppliers. Tyrosine, dihydroxyphenylalanine (DOPA), dopamine (DA), norepinephrine (NE), dihydroxyphenylacetic acid (DOPAC), dihydroxyphenylglycol (DOPEG), 3-methoxy-4-hydroxyphenylglycol (MOPEG), homovanillic acid (HVA), tyramine, octopamine, leucine, choline, phosphorylcholine and cytidine diphosphorylcholine were obtained from Sigma Chemical Co., Saint Louis, Mo. Amphetamine and p-hydroxyamphetamine were the generous gift of Smith, Kline and French Laboratories, Pheladelphia, Pa. Para-hydroxynorephedrine (PONE) and norephedrine were purchased from Aldrich Chemical Co., Chicago, Ill. Standard solutions of phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), phosphatidylinositol (mono) (PI), and phosphatidylserine (PS) were obtained from Supelco. Inc., Bellfonta, Pa.

$^3$H-Tyrosine (2,3 side chain (11 to 15 Ci/mmol), $^{32}$P-phosphoric acid (carrier free), $^3$H-DA (2 side chain) (10 Ci/mmol), $^3$H-*ℓ*-NE (2 side chain) (6-8 Ci/mole), $^3$H-*d*-amphetamine (general label) (1 Ci/mmol), $^3$H-leucine (4,5-$^3$H) (55 Ci/mmol) and $^3$H-γ-aminobutyric acid (general label) (0.3 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass. $^{14}$C-Choline (methyl-$^{14}$C) (0.5 Ci/mmol), $^3$H-choline (methyl-$^3$H) (13 to 15 Ci/mmole), $^3$H-NE (2 side chain) (8-10 Ci/mmole) and $^3$H-serotonin (general label) (10 Ci/mmole) were obtained from Amersham-Searle Corp., Chicago, Ill. Trypsin (2X crystallized, salt free, 10,000 BAEE units/mg solid), phospholipase A (Vipera russelli, 4 units/mg solid) and phospholipase C (Cl. welchii 2 units/mg solid) were obtained from Sigma Chemical Co. Enzyme purity was checked by disc gel electrophoresis (7.5% gel, Coomassie Blue Dye) but further enzyme purifications were not performed. Only trypsin yielded a single band upon electrophoretic separation. Contaminating enzymes in the phospholipase A solution were denatured by immersing the solution in boiling water for 7 minutes.

## 3.3 PREPARATION OF SPECIAL REAGENTS.

Biorad AG-50X-4, 400 mesh $^+$H form resin was used for purification of catecholamines and tyrosine. Unlike the earlier Dowex resins, the Biorad resin is generally of sufficient quality as not to require further purification. However, as a precaution, the resin was washed in the following manner. Using a large Büchner funnel with two layers of Watman #1 filter circles, 200 to 300 g of resin was first washed with copious amounts of 2 N NaOH plus 1% EDTA. NaOH was removed with several washes of distilled $H_2O$. The resin now in the $Na^+$ form was changed back to the $H^+$ form by washing with 3 N HCl. Excess acid was removed by several washes with distilled $H_2O$ until the pH of the wash was between 4 and 5. The resin was stored in several volumes of distilled $H_2O$ until use.

Acid activated alumina (Anton and Sayre, 1962) used for the extraction of catecholamines was prepared as follows. One hundred to 200 grams of neutral alumina (Fischer Chemical Co.) was suspended in 1000 ml of 2 N HCl in a large beaker. The beaker was covered with an evaporating dish and heated to boiling with constant stirring. The boiling was continued for 1 to 2 hours or until the suspension turned yellow. The boiling treatment was stopped and exactly 1 minute later the acid was carefully decanted off. The alumina was resuspended in 1 N HCl and heated at 75° C with constant stirring for 1 hour. As before, the acid was decanted away after letting the solution stand for 1 minute. The alumina was then washed with several changes of distilled $H_2O$ until the pH of the wash was approximately 5. The alumina was dried at 120° C for 2 to 3 hours and then stored in a dry, cool place until use.

Two scintillation cocktail solutions, chosen for their ability to hold large amounts (1-3 ml) of aqueous sample, were used. Modified Bray's solution (Bray, 1960) contained 60 grams napthalene, 100 ml spectrophotometric grade methanol, 4 grams PPO, 50 milligrams POPOP and scintillation grade dioxane to make 1 liter. The other cocktail used was a Toluene-Triton X-100 solution containing 2 parts toluene plus 6 grams PPO and 1 part Triton X-100 (Rohm and Haas, Philadelphia, Pa.). Using a Beckman LS-100 scintillation counter, the efficiency of both cocktails was 10 to 25% for $^3H$-samples depending on the amount of $H_2O$ added. The amount of $H_2O$ in the toluene-triton cocktail should be greater than 10% or less than 5% by volume. In the 5 to 10% range, a milky precipitate often forms which can be dissolved by adding more $H_2O$.

For *in vitro* tissue slice, homogenate or nerve ending particle studies, either a modified Krebs-Ringer-Bicarbonate (KRB) or Krebs-Ringer-Phosphate (KRP) salt solution was used. The standard KRB buffer contained 128 mM CaCl, 32 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 0.75 mM $CaCl_2$ and 10 mM glucose. The solution was bubbled with 95% $O_2$ - 5% $Co_2$ at 37° C for 30 min and the final pH was adjusted with 0.1 NaOH to 7.4. The KRP buffer as described by Harris and Roth (1971) contained 128 mM CaCl, 4.8 mM KCl, 0.75 mM $CaCl_2$, 16 mM $Na_2HPO_4$ and 10 mM glucose. As with the KRB buffer, the KRP buffer was bubbled with 95% $O_2$ - 5% $CO_2$ at 37° C for 30 min and then adjusted to a pH of 7.4. The KRP buffer was then bubbled again with the $O_2$ - $CO_2$ mixture for an additional 30 min before making a final small pH adjustment. In some experiments the KRB buffer described by Katz and Kopin (1969) containing the additional energy sources pyruvate, glutamate and lactate was used.

## 3.4 PREPARATION OF SPECIAL TISSUE SAMPLES.

Tissue slices were prepared in the following manner. Animals were sacrificed, brains rapidly removed and dissected. The tissue was then placed in an ice-cold buffer to cool for 2 to 3 min. The cooled tissue was sliced using a McIlwain tissue chopper set at a thickness of 0.26 mm. The slices were rapidly transferred to incubation vesicles containing buffer constantly bubbled with a mixture of 95% $O_2$ - 5% $CO_2$ at 37° C.

Nerve ending particles (NEP) were prepared by two methods. In both methods sucrose or ficoll solutions were buffered to pH = 7.0 using 0.5 mM N-2 hydroxyethylpiperazine-N$^1$-2-ethanesulfonic acid (HEPES). The method of von Hungen *et al.* (1968) was used for the experiments described in Chapter 8. Cortical tissue (2 to 3 grams) was homogenized in 9 vol of 0.32 M sucrose with a loosely fitting teflon-glass homogenizer using 8 to 10 excursions. For optimal results, the speed of the homogenizer should be 750 to 1000 rpm (Whittaker, 1969). The homogenate was centrifuged 10 min x 750 g in a refrigerated centrifuge. The supernatant was then centrifuged 20 min x 17,000 g. The supernatant obtained can be further processed by centrifugation for 1 hour x 100,000 g to obtain the microsomal pellet and the soluble fraction. The crude mitochondrial pellet containing mitochondria, myelin and NEP obtained from the 17,000 x g centrifugation was resuspended in 8 ml of 0.32 M sucrose. This suspension was then layered on a discontinuous sucrose gradient containing 10 ml 1.2 M, 5 ml 1.1 M and 10 ml 0.8 M sucrose. The gradient was centrifuged for 1 hour x 25,000 rpm (53,000 g) using a Beckman SW 25 swinging bucket rotor. Morphological analysis indicated that NEP were located in the 1.1 M sucrose band. The purity of the NEP was estimated to be 60 to 70 percent on the basis of electron micrographs.[1] The NEP in the 1.1 M sucrose band were diluted with an equal volume of 0.32 M sucrose and then centrifuged for 1 hr x 100,000 g. Pelleted NEP were then resuspended in the appropriate buffer for experimental use.

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[1] Electron micrographs were kindly prepared by Dr. Robert Lee, USCF Department of Pathology. Tissue was fixed in glutaraldehyde, stained with osmium tetroxide and postained with uranyl acetate and lead citrate.

The second procedure used for the isolating of NEP and synaptic plasma membranes (SPM) is illustrated in Fig. 11. The reasons for processing cortical and subcortical[2] tissue in a different manner was as follows. It seemed reasonable to predict that amphetamine will have different effects on the protein and phospholipid turnover of NEP depending on the type of NEP examined (see Chapter 10). Kuhar *et al.* (1970) have demonstrated in some subcortical regions that two populations of NEP can be differentiated on the basis of their relative ability to concentrate labeled NE and GABA. These authors incubated a crude homogenate with a mixture of $^{3}$H-GABA and $^{14}$C-NE and upon centrifugation of the homogenate in a continuous sucrose gradient, separate $^{3}$H and $^{14}$C peaks were obtained in the region where NEP sediment. This same approach was employed to find a discontinuous sucrose gradient that would separate GABA and NE containing NEP. The gradient shown in Fig. 7 was found to be adequate for this purpose. When a crude mitochondrial suspension is incubated with $^{14}$C-NE and $^{3}$H-GABA, pelleted, washed, resuspended and applied to this gradient, a separation of $^{3}$H-GABA and $^{14}$C-NE in the region containing the NEP is obtained. The same procedure was tried using a crude mitochondrial suspension derived from cortical tissue. However, the $^{3}$H-GABA and $^{14}$C-NE peaks did not separate well. Alternative gradients, different centrifugation speeds and different centrifugation times were tried but none were effective when cortical tissue was used. The discontinuous ficoll gradient finally adopted for use with cortical tissue was developed by Cotman and

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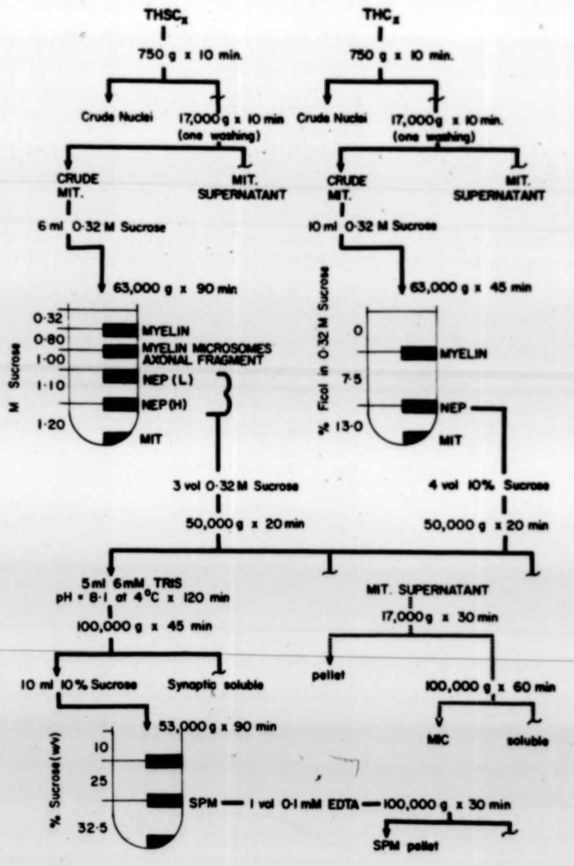
[2] The subcortex is defined as that portion of the brain anterior to the inferior colliculi with the cerebral hemispheres removed.

Fig. 11. Flow sheet for the preparation of SPM from different populations of rat brain NEP. Legend - $THSC_X$ = total homogenate subcortex; $THC_X$ = total homogenate cortex; MIT = mitochondria; MIC = microsomes; SPM = synaptic plasma membranes; NEP = nerve ending particles (light [L]) or (heavy [H]). The NEP(L) were isolated from that region of the gradient which concentrates $^3$H-GABA and the NEP(H) were isolated from that region which concentrates $^{14}$C-NE (see Fig. 7 for details).


THSC$_x$
750 g x 10 min.
Crude Nuclei
17,000 g x 10 min
(one washing)
CRUDE MIT.
MIT. SUPERNATANT
6 ml 0·32 M Sucrose
63,000 g x 90 min
M Sucrose
0·32
0·80
1·00
1·10
1·20
MYELIN
MYELIN MICROSOMES
AXONAL FRAGMENT
NEP (L)
NEP (H)
MIT
THC$_x$
750 g x 10 min.
Crude Nuclei
17,000 g x 10 min.
(one washing)
CRUDE MIT.
MIT. SUPERNATANT
10 ml 0·32 M Sucrose
63,000 g x 45 min
% Ficol in 0·32 M Sucrose
0
7·5
13·0
MYELIN
NEP
MIT
3 vol 0·32 M Sucrose
50,000 g x 20 min
4 vol 10% Sucrose
50,000 g x 20 min
5 ml 6 mM TRIS
pH = 8·1 at 4°C x 120 min
100,000 g x 45 min
10 ml 10% Sucrose
Synaptic soluble
53,000 g x 90 min
% Sucrose (w/v)
10
25
32·5
SPM — 1 vol 0·1 mM EDTA — 100,000 g x 30 min
SPM pellet
MIT. SUPERNATANT
17,000 g x 30 min
pellet
100,000 g x 60 min
MIC
soluble

Matthews (1971). The ficoll gradient has some advantages over sucrose gradients in that (1) a higher yield of SPM is obtained, (2) the microsomal contamination is reduced, (3) the NEP don't shrink as they do with hypertonic sucrose gradients. Mitochondrial contamination of NEP prepared on ficoll gradients is generally higher than with sucrose gradients. Since the NEP isolated from the discontinuous sucrose gradient cannot be considered to consist of purely NE or GABA NEP, the GABA NEP are referred to as NEP-light (L) and the NE NEP are referred to as NEP-heavy (H).

The preparation of SPM from the three populations of NEP was as described by Cotman and Matthews (1971). The purity of the SPM fractions was determined by using various enzyme markers as suggested by Mahler and Cotman (1970) and by ganglioside enrichment (Morgan *et al.*, 1971). Enzyme marker assays were performed in the following manner.

Na$^+$, K$^+$ activated, ouabain-sensitive ATPase (Mahler and Cotman, 1970). Enzyme activity was assayed by determining the difference between the amount of ATP hydrolyzed in the presence and absence of ouabain. The assay mixture contained 1 ml of buffer which consisted of 50 mM Tris, 100 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$ with or without 0.2 mM ouabain at pH 7.3 and 30 to 100 μg of protein in volume of 0.1 ml. The mixture was preincubated for 10 min and then 0.05 ml of 60 mM ATP was added. The reaction was terminated after 5 min by adding 5 ml of an aqueous solution containing 0.2% ammonium molybdate and 0.2% $H_2SO_4$. Color was developed by adding 0.2 ml of a solution containing 6 g Na sulfite, 30 g Na bisulfite and 0.5 g 1-amino-2 napthol-4-sulfonic acid in 250 ml of $H_2O$. Optical density was read at 600 nm.

Antimycin-sensitive NADH oxidase (Mahler and Cotman, 1970). Enzyme activity was assayed by following the oxidation of NADH at 350 nm, the isobestic point of cytochrome C. The assay mixture contained 1 ml 0.05 M sodium phosphate buffer pH = 7.3, 0.05 ml NADH (1 mg/ml), 0.02 ml cytochrome C (10 mg/ml), 0.012 ml antimycin C (100 µg/ml in ethanol) and 20 to 50 µg of enzyme in 0.1 ml. The reaction was started by adding NADH. OD measurements were made every minute for 5 min.

Cytochromc C oxidase (Duncan and Mackler, 1966). Enzyme activity was assayed by following the oxidation of cytochrome C at 550 nm. The assay mixture contained 0.2 ml 0.2 M phosphate buffer (pH = 7.5), 0.1 ml 1% reduced cytochrome C, enzyme - 5 to 10 µg and $H_2O$ to 1 ml.

Ganglioside enrichment (Suzuki, 1965; Warren, 1959). Tissue suspended in 0.5 ml 0.1 N KCl was extracted with 8 ml of chloroform-methanol (2:1,v/v) followed by extraction with chloroform-methanol (1:2,v/v). The extracts were combined and chloroform added to bring the chloroform-methanol ratio to 2/1. The organic phase was successively partitioned with 0.2 vol 0.1 N KCl, theoretical upper phase (47% 0.1 N KCl, 48% MeOH, 3% chloroform) and theoretical upper phase with $H_2O$. The aqueous phases were combined, dialized and concentrated to 1/5 of their original volume.

Sialic (N-acetylneuraminic) acid content in the ganglioside fraction was determined essentially by the method of Warren (1959). 0.1 ml of the aqueous solution was mixed with 0.1 ml of 0.2 N $H_2SO_4$ and the sample was incubated at 80° C for 1 hr in a 15 ml conical centrifuge tube capped with a glass marble. After cooling, 0.2 ml periodate solution (0.2 m meta-sodium perrodate in 4.5 M phosphoric acid) was added and the solution was allowed to stand for 20 min. Then 1 ml of arsenite reagent (10% Na arsenite in 0.5 M Na sulfate containing 0.1 N $H_2SO_4$) was added and the solution was mixed on a Vortex mixer until the yellow-brown color disappeared. Three ml of thiobarbituric acid solution (0.6% thiobarbituric acid in 0.5 M Na sulfate) was then added, the tubes were capped with a marble and heated in a boiling water bath for 15 min. The tubes were cooled in tap water for 5 min during which time the red-pink product fades and becomes cloudy. The solution was extracted with 3 ml acid butanol (butanol:HCl, 95:5, v:v), centrifuged and the organic phase was read at 549 nm. Corrections for tissue and 2-deoxyribose contamination were suggested by Warren (1959).

A comparison of the relative enrichment for the various markers in the SPM, microsomal and mitochondrial fractions is shown in Table 5. The data indicate the SPM fractions were 85 to 90 percent pure on the basis of enrichment in ATPase activity and on the basis of contamination with microsomal and mitochondrial tissue as measured by NADH oxidase and cytochrome C oxidase activity, respectively.

## 3.5 EXTRACTION OF PHOSPHOLIPIDS.

Phospholipids were extracted from undenatured and denatured (usually TCA treated) tissue essentially as described by Abdel-Latif and Smith (1969). Tissue (400 mg wet weight or less) was extracted with 8 ml chloroform-methanol-HCl (200:100:1 v/v/v) containing 0.05 grams/100 ml α-tocopherol succinate. The sample was incubated for 15 to 20 min at 50° C and then allowed to stand overnight in a refrigerator. The following day the sample was centrifuged for 10 min x 10,000 g. The organic phase was removed and partitioned twice with 0.2 vol of 0.9%

## TABLE 5

## Comparison of Enzyme-Specific Activities in Various Fractions [a]

| | Relative Enzyme Activity | | | | |
|---|---|---|---|---|---|
| Fraction | $Na^+$, $K^+$ ATPase | Antimycin Insensitive NADH-Cytochrome Oxidase | MAO | Cytochrome Oxidase | Gangliosides |
| TH | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Mit | - | 2.0<br>(1.8) | 6.0<br>(5.3) | 6.2<br>(7.2) | - |
| Mic | - | 3.9<br>(4.2) | - | - | - |
| SPM-L | 7.5 | 0.4 | 2.8 | 0.3 | 5.2 |
| SPM-H | 8.5 | 0.3 | 2.7 | 0.4 | 6.3 |
| SPM-$C_X$ | 7.2 | 0.3 | 1.9 | 0.3 | 5.7 |

[a] All data are compared to that in the total homogenate on an activity/mg protein basis. Data are the mean values obtained from five experiments. $Na^+$, $K^+$, ATPase and NADH oxidase were assayed as described by Mahler and Cotman (1970). Cytochrome oxidase was determined by the method of Duncan and Mackler (1966). Gangliosides were determined as described by Brunngraber *et al.* (1971). The values in parentheses are from the cortex. Data from the SPM fraction on NADH oxidase was corrected for contamination by mitochondrial NADH oxidase based on the ratio of cytochrome oxidase/NADH release in the two fractions. Abbreviations used: TH - total homogenate; Mit - mitochondria; Mic - microsomes; SPM - synaptic plasma membrane; SPM-L - SPM derived from light nerve endings of subcortex; SPM-H - SPM derived from heavy nerve endings of subcortex; SPM-$C_X$ - SPM derived from cortical nerve endings.

saline. The aqueous layer was carefully removed by aspiration after each partition. Following this treatment, the organic phase is evaporated to dryness overnight at 40 to 45° C. Depending on the original amount of tissue, the residue is dissolved in 0.15 to 0.50 ml of chloroform. In duplicate, 50 µl of the chloroform extract is applied to a thin layer Silica Gel G plate (thickness 250 microns) for two dimensional chromatography. The solvent system for the first direction was chloroform-methanol-acetic acid-$H_2O$ (25:15:4:2, v/v/v/v); upper phase butanol-pyridine-$H_2O$ (9:1:4, v/v/v) was used for the second dimension. Fig. 12 pictorially illustrates the separation of the various phospholipids that is obtained with this procedure.

The various phospholipid spots were carefully scraped into 15 ml conical centrifuge tubes containing 0.2 ml of distilled $H_2O$ to wet the silica gel. Phospholipids were extracted from the silica gel with 5 ml of chloroform-methanol (1:1, v/v) by shaking 20 sec with a Vortex mixer, incubating the samples for 1 hour at 50° C and finally by mixing the samples with the Vortex mixer. The samples were centrifuged at 1,000 rpm on a table model ICN clinical centrifuge (approximately 200 g). The organic extract was then divided into two or more portions for radioactivity and phosphorus determinations.

Conveniently, 1 to 2 ml of the organic extract was pipetted into glass scintillation vials. The sample was dried at 40 to 50° C prior to the addition of 5 ml of triton-toluene cocktail.

Lipid bound phosphorus was determined by the method of Bartlett (1959). One, 2 or 3 ml of sample (duplicate samples done when possible) was placed in 0.8 x 15 cm test tubes and dried at 40 to 50° C. Then 0.4 ml of 70% perchloric acid was added to each sample prior to

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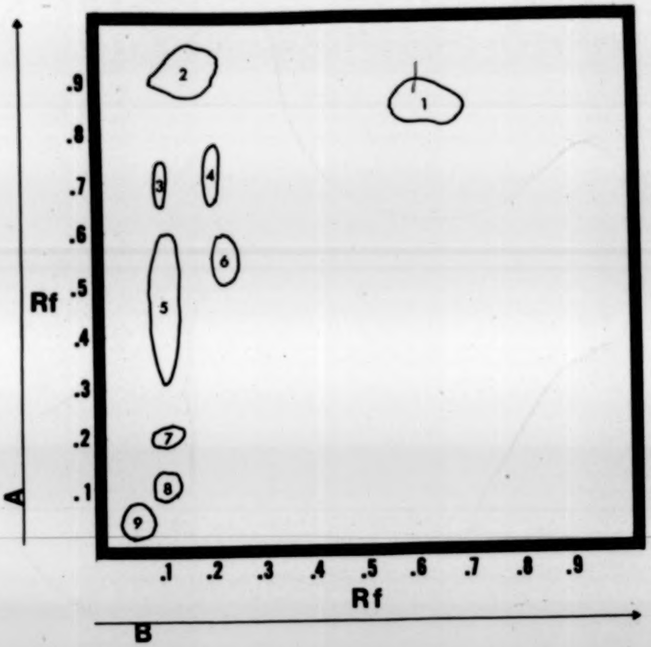
Fig. 12. Two dimensional separation of phospholipids on Silica Gel G thin layer plates. The first solvent system (A) consisted of chloroform/methanol/acetic acid/$H_2O$ (25:15:4:2) and the second system (B) of upper phase butanol/pyridine/$H_2O$ (9:1:4). Legend: 1 = phosphatidylethanolamine; 2 = phosphatidic acid; 3 = phosphatidylserine; 4 = phosphatidylinositol; 5 = phosphatidylcholine; 6 = lysophosphatidylethanolamine; 7 = lysophosphatidylcholine; 8 = sphingomyelin; 9 = origin.

XUM


1
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hydrolysis for 2 hr x 200° C. A heating block which only comes in contact with the bottom half of the test tube is convenient for this purpose. After cooling, 2.4 ml of ammonium molybdate solution (4.4 g of ammonium molybdate plus 14 ml concentrated $H_2SO_4$ to 1 liter with $H_2O$) was added, followed by 2.4 ml of dilute Fiske-Subarow reagent (2.5 g Na bisulfite, 0.5 g Na sulfite and 42 mg 1,2,4 amihonapthol sulfonic acid in 250 ml $H_2O$). The samples were mixed and then heated in a boiling water bath for 10 min. The blue color was read at 750 nm.

### 3.6 $^{14}$C-CHOLINE METABOLISM.

For some of the studies described in Chapter 9 it was decided to examine the cerebral metabolism of $^{14}$C-choline. Brain tissue was homogenized in 10 vol of 10% TCA and centrifuged. The supernatant was extracted four times with diethyl ether to remove the TCA and then evaporated to dryness. The residue was resuspended in 100 μl of MeOH-HCl (20:1, v/v) containing 10 μg each choline, phosphorylcholine and cytidine diphosphorylcholine choline. Fifty μl of the extract was spotted on cellulose thin-layer plates (Gilman Instrument Co., MN-300). Plates were developed using a solvent system of n-butanol, ethanol, acetic acid and water (8:2:1:3, v/v/v/v) (Gomez *et al.*, 1971). Spots were visualized with dilute iodine spray (1% $I_2$ in MeOH). After sublimation, the spots were scraped into scintillation vials containing 1 ml MeOH-HCl (20:1, v/v). The samples were allowed to stand overnight before adding 10 ml of modified Bray's cocktail.

### 3.7 LABELED AND UNLABELED PROTEIN DETERMINATION.

The determination of labeled protein was by a modification of the method previously used by Loh *et al.* (1971). 0.1 to 0.3 ml of protein suspension was added to 2 ml of 10% TCA plus 1% unlabeled amino acid. Samples were then incubated at 80° C for 30 min. Using a millipore filtering apparatus, protein was collected on GFA or GFB Whatman fiberglass filters. The protein was then washed successively with 10 ml each of 10% TCA plus 1% unlabeled amino acid, chloroform-methanol (2:1, v/v) and methanol. Filters were placed in scintillation vials and 10 ml of toluene-triton cocktail was added. Samples were allowed to stand overnight before counting.

Endogenous protein was determined by a modification of the method of Lowry *et al.* (1951). Ten to 100 µl of protein suspension containing 10 to 50 µg of protein was mixed with 0.8 ml 0.1 N NaOH. The mixture was incubated for 1 hour x 45° C. After cooling, 2 ml of a freshly prepared solution containing cupric sulfate (0.01%), potassium tartrate (0.02%), 0.1 N NaOH and sodium carbonate (2%) was added. The samples were mixed and allowed to stand for 10 min. 0.2 ml of 1 N phenol reagent, diluted from 2 N with $H_2O$, was then added to each sample with immediate mixing. Samples were allowed to stand for 30 min before reading absorbancy at 700 nm. Standard solutions were made with bovine serum albumin.

## 3.8 CATECHOLAMINE, CATECHOLAMINE METABOLITE AND TYROSINE DETERMINATION.

Regardless of the experimental design, all studies where a determination of endogenous or labeled catecholamines and related substances was required began by homogenizing the tissue in approximately 10 volumes (w/v) of 0.4 N $HClO_4$ and centrifuging for 10 min x 10,000 g. The pH of the perchloric acid extract was adjusted to 8.2 to 8.4 with 2 N Tris and the mixture was then added to a 30 ml Nalge polypropylene Oak-Ridge shaking tube containing 0.5 g of activated alumina. After shaking for 10 min, the samples were centrifuged for 10 min x 2,000 g. The supernatant was carefully decanted off, frozen and saved for further analysis. Before eluting the catechols from the alumina, it was found necessary to wash the alumina at least three times with 0.2 N Tris (pH = 8.3) in those experiments where $^3$H-tyrosine was used. If this washing procedure was not performed, it was found that the $^3$H-tyrosine trapped in the alumina pellet greatly interfered with the determination of $^3$H-DA and $^3$H-NE. Catechols were eluted from the alumina with 5 ml of 0.2 N acetic acid. The elute was then divided into 1 and 3 ml portions. The 1 ml portion was analyzed for endogenous DA and NE by the method of Ansell and Beeson (1968). This procedure is a modification of the standard trihydroxyindole method described by several workers (see Udenfriend, 1962). To the acetic acid elute, 0.2 ml of 0.1 N $I_2$ in ethanol was added. Exactly 2 min later 0.4 ml of alkaline sulfite solution (1.0 N Na bisulfite:5 N NaOH, 1:9, v/v) was added. Exactly 1.5 min later 0.4 ml of 6 N acetic acid was added. The samples were placed in a boiling water bath for 2 min and then cooled in tap water. NE was read on an Aminco-Bowman spectrophotofluorometer at 385 nm activation - 485 nm excitation. After reading for NE, the samples were returned to the boiling water bath for an additional 10 min. DA was read, preferably the next day, at 335 nm activation - 385 nm excitation. NE interfered with the determination of DA only very slightly; the contamination was corrected for by running appropriate standards. Tissue standards and blanks were used in all determinations. The analysis of DA and NE from the acidic acid elute rather than from the column elute (see below) was found to be more satisfactory for several reasons: (a) the blank was considerably lower; (b) it was possible to add cold carrier NE and DA to the columns which greatly improved the separations obtained, and (c) the necessity of making tedious pH adjustments to strongly acidic elutes was eliminated.

The 3 ml portion of the alumina elute was adjusted to pH 5.0 - 5.2 by adding 1 ml of 2 N sodium acetate containing 100 μg each of carrier NE and DA. This solution was then analyzed for $^3$H-DA and $^3$H-NE by column chromatography as described by Taylor and Laverty (1969). Vitamin A tubes (Scientific Glass Apparatus Co.) were plugged with Pyrex glass wool and sufficient AG-50 resin was added to make a 5 cm column. After washing the column with 20 ml of distilled $H_2O$, the sample was applied. The column was again washed with 10 to 30 ml of $H_2O$ prior to elution. The elution of NE and DA was as follows: 3 ml of 1.2 N HCl was applied to the column and the elute obtained discarded. NE was then eluted with 3 ml of 1.2 N HCl. DA was eluted with 6 ml of 2.0 N HCl. The percent recovery of DA and NE from the columns was in general found to be 70 and 85 percent respectively. Portions (2 ml) of the acid elutes were transferred to scintillation vials containing 15 ml toluene-triton cocktail. Counting efficiency in the LS-100 as determined by both internal and external standards was in the range of 10 to 12 percent. The columns used to separate DA and NE were washed with 20 ml of 3 N HCl and then with sufficient $H_2O$ until the pH of the elute was approximately 5. These columns were then used for the determination of tyrosine.

Endogenous and labeled tyrosine were isolated by the method of Weiner and Rabadjija (1968). The pH of a 2 to 4 ml portion of the alumina effluent was adjusted to 2 and this solution was applied to the AG-50 column. After washing the column with 20 ml 0.1% EDTA and

10 ml of distilled $H_2O$, tyrosine was eluted with 5 ml of 1 N $NH_4OH$. One ml of the $NH_4OH$ elute was neutralized with acetic acid and then counted 15 ml of Bray's solution. Endogenous tyrosine was determined by the nitroso-napthanol method of Wong *et al.* (1964). 0.5 ml of the $NH_4OH$ elute was neutralized with 0.1 ml of 50% TCA before the addition of 6 ml of nitroso-napthol reagent. The reagent consisted of α-nitroso-β-napthol (200 mg/100 ml 95% ethanol); 3.0 N $HNO_3$ and 0.1 N Na nitrite (2:3:3, v/v/v). The mixture was then incubated for 20 min at 33° C. After incubation, the mixture was extracted with 12 ml ethylene dichloride and the upper aqueous phase was saved. The aqueous phase was then allowed to stand at room temperature for 30 to 45 min before reading on the spectrophotofluorometer at 460 nm activation - 485 nm excitation.

Preliminary experiments indicated that when $^3H$-tyrosine was used as the substrate, the levels of the labeled 3-methoxylated catecholamines and the labeled neutral and acidic catechols were not sufficiently above background to warrant further investigation. Interestingly, thin layer chromatographic analysis of those column fractions containing normetanephrine and 3-methoxytyramine obtained by the Taylor and Laverty (1969) method indicated that a majority of the radioactivity was not in these compounds but rather in tyramine and octopamine. It was, however, possible to accurately determine the levels of $^3H$-3-methoxy-4-hydroxyphenylglycol (MOPEG) and $^3H$-homovanillic acid (HVA) and the procedures used will be described in some detail. That portion of the alumina effluent not used for tyrosine analysis was extracted with 5 volumes of ethyl acetate at both pH = 7 and pH = 1.5. The ethyl acetate extracts were combined and evaporated to dryness. The residue was redissolved in 1 ml of 1 N sodium acetate pH = 5 containing 10 units of glucoronidase-sulfatase (Sigma Chemical Co.) and incubated for 1 hr x 37° C. MOPEG and HVA were then extracted into ethylacetate as previously described. The extracts were combined, evaporated to dryness and the residue dissolved in 100 μl of methanol-HCl (20:1, v/v). Fifty μl of the extract was spotted on silica gel impregnated paper (Brinkman Instrument Co.) and the metabolites were separated in a solvent system of benzene:glacial acetic acid (50:3, g/g). Spots were visualized with a dilute iodine solution and after sublimation the spots were cut out and placed in scintillation vials containing 0.5 ml methanol. The samples were extracted overnight before adding 10 ml of Bray's counting solution. Percent recovery was determined by carrying standard solutions of $^3$H-MOPEG and $^3$H-HVA through the procedures outlined above. The recoveries of both HVA and MOPEG were generally in the range of 40 to 50 percent.

## 3.9 CATECHOLAMINE UPTAKE.

The uptake of catecholamines was variously determined in brain slices, homogenates or purified nerve ending particles. However, the procedures involved do not differ greatly from one type of preparation to another. Brain tissue (slice, homogenate, NEP) was prepared as previously described. The tissue was transferred to reaction vesicles containing 4.0 ml of KRB buffer and preincubated for 5 min at 37° C. After preincubation, drugs are added in a volume of 0.5 ml and the incubation continued for 5 min. Various concentrations of high specific activity $^3$H-NE or $^3$H-DA (final concentration $2 \times 10^{-7}$ M to $10^{-8}$ M) were added in a volume of 0.5 ml so that the final reaction volume was 5 ml. In these studies only the initial rapid and reserpine insensitive (Snyder and Coyle, 1969) uptake was examined so that after adding the $^3$H-catecholamine the reaction was terminated either at 2.5 or 5 min by immersing the samples in an ice water bath. The additional advantage of using a short incubation time is that it is not necessary to add a MAO inhibitor to the reaction mixture. When tissue slices were used, the slices were washed several times with ice-cold buffer using the GFA filters and the Millipore filtering apparatus. The slices were then carefully scraped from the filter paper, weighed, homogenized in 3.0 ml of 0.4 N $HClO_4$ and centrifuged for 10 min x 10,000 g. Duplicate aliquots of the supernatant were counted in 5 ml of Bray's cocktail. When tissue homogenates or NEP were used, the cooled samples were centrifuged for 10 min x 45,000 g. The supernatant was discarded and the pellet was washed once with ice-cold buffer. The final pellet was homogenized in 3 ml $HClO_4$ as with the slices. Snyder and Coyle (1969) have shown under nearly identical incubations conditions that uptake was linear with time when the incubation period was 5 min or less. Recently some improvement in the technique for measuring uptake in the homogenate or NEP has been attempted. The reaction was terminated by rapid filtration using GFB filters (pore size 1 μ) and then washing the filters several times with ice-cold buffer. As judged by the amount of protein appearing in the effluent, it appears there is little if any loss of tissue through this filter.

### 3.10 CATECHOLAMINE SPONTANEOUS RELEASE.

The spontaneous release of $^3H$-catecholamines from tissues preloaded with $^3H$-catecholamine was measured using both tissue slices and a crude mitochondrial fraction. The procedure for tissue slices was as follows. Slices (0.26 mm) were prepared from either the cortex or corpus striatum. Approximately 100 mg wet weight of slices was dispersed in 5 ml of modified KRB buffer (Katz and Kopin, 1967) in 50 ml round-bottom glass centrifuge tubes. Slices were preincubated for 10 min at 37° C and bubbled with 95% $O_2$ - 5% $CO_2$ throughout all the procedures. Slices were then incubated with $^3$H-NE ($1 \times 10^{-7}$ M), $^3$H-DA ($1 \times 10^{-6}$ M) or $^3$H-tyrosine ($1 \times 10^{-7}$ M) for 20 min. After incubation, the slices were lightly centrifuged for 10 min x 300 g and the supernatant was discarded. The slices were washed twice with ice-cold KRB buffer and finally incubated in 5 ml of fresh medium. Preliminary experiments indicated that the release of radioactivity to the medium increased linearly during the initial 20 min of incubation and subsequently a 20 min incubation was used in all experiments. The incubation was terminated by cooling the tubes in an ice-bath and followed by centrifugation for 10 min x 1,000 g. The supernatant was carefully decanted away, acidified with 2 ml of 0.4 N $HClO_4$ and frozen at -20° C for subsequent analysis. The slices were homogenized in 3 ml of 0.4 N $HClO_4$ and centrifuged for 10 min x 10,000 g. The pellets were rehomogenized in 4 ml distilled water and supernatants were pooled. The supernatant was frozen until analysis, which was performed within 48 hr. Prior to freezing, an 0.1 ml sample of both the medium and tissue extract was counted in 5 ml of toluene-triton cocktail.

Alternatively to the procedure described, slices preloaded with $^3$H-NE or $^3$H-DA were transferred to vitamin A tubes plugged with Pyrex glass wool. Samples were then superfused with KRB buffer maintained at 37° C and saturated with 95% $O_2$ - 5% $CO_2$. Five ml fractions of superfusion medium were collected and a portion (0.5 ml) of each fraction was analyzed for total radioactivity.

In both the normal and superfusion release, aliquots of the tissue and medium perchlorate extracts were analyzed for $^{3}$H-catecholamines and $^{3}$H-catecholamine metabolites by the procedures previously described.

The logic behind using a crude mitochondrial fraction to measure spontaneous release was the same as for using this fraction to measure uptake; labeled catecholamines are almost entirely sequestered within the NEP of a crude mitochondrial fraction. In the procedure to be described, all animals were pretreated with 75 mg/kg i.p. of the MAO inhibitor pargyline 20 to 24 hr prior to sacrifice. The crude mitochondrial pellet (30 to 40 mg protein) from 1 gram of cortical tissue was suspended in 40 ml of KRB buffer and preincubated for 10 min at 37° C. Sufficient $^{3}$H-NE was then added to bring the final NE concentration to $10^{-7}$ M and the incubation was continued for 10 min. The reaction was terminated by centrifugation (10 min x 48,000 g) and the pellet obtained was washed twice with ice-cold buffer. The final pellet was resuspended in 40 ml of ice-cold buffer and divided into 10 ml portions. One portion was kept in ice water throughout the procedure and served as the blank. To the other portions drugs, enzymes, etc., were added depending on the experimental design. The reaction was begun by placing the reaction vesicle in a shaking water bath at 37° C. Two ml aliquots were removed at various times up till 60 min and immediately centrifuged for 10 min x 48,000 g. 0.5 ml aliquots of the supernatant were counted in 10 ml of Bray's cocktail. Chromatographic analysis indicated that more than 90% of the released $^{3}$H was in the form of $^{3}$H-NE.

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## 3.11 *In vitro* CATECHOLAMINE SYNTHESIS.

*In vitro* catecholamine synthesis in brain tissue slices was performed as described by Harris and Roth (1971) with little modification. Tissue slices (50 to 100 mg) prepared from the cortex, brain stem or caudate nucleus were prepared as previously described. The slices were transferred to incubation flasks containing 9 ml of KRP buffer bubbled with 95% $O_2$ - 5% $CO_2$. After a 5 min preincubation, 1 ml $^3$H-tyrosine (10 μCi/2 x $10^{-4}$ M) was added and the incubation was continued for 30 min. Harris and Roth (1971) have shown that with the amount of tissue used, 2 x $10^{-5}$ M tyrosine is a saturating concentration and that the synthesis of labeled catecholamines is linear for at least 30 min. The reaction was terminated by cooling the reaction flasks in an ice-water bath, followed by light centrifugation (10 min x 200 g). Five ml of the supernatant was removed, acidified with 2.4 N $HClO_4$ and frozen until analysis. The remainder of the supernatant was carefully discarded and the slices were washed once with 10 ml of cold KRP buffer. The slices were lightly blotted dry, weighed and homogenized in 4 ml 0.4 N $HClO_4$. The sample was centrifuged (10 min x 10,000 g) and the supernatant frozen until analysis.

## 3.12 LOCOMOTOR ACTIVITY AND STEREOTYPED BEHAVIOR.

Locomotor activity was measured by two methods. The first method employed the Pharmacia MP 40 Fc motility meter. This meter contains 40 photocells arranged in 5 rows of 8 covered with a thin plexiglass sheet. A standard laboratory mouse cage exactly fits the photocell design. The photocells require no additional lighting source except standard laboratory lighting. One count is registered for every photocell contact broken. Cumulative counts are registered on a digital meter contained in the machine. Additionally, the motility meter was connected to a chart recorder to obtain a temporal activity profile. Animals were acclimated to clear plastic laboratory cages without bedding material for at least 1 hour prior to running the experiment. In order to minimize the measurement of stereotyped activities with the motility meter (see below), in some experiments all the photocells were removed except for one medial row of five.

During the course of the experiments described in this dissertation, it became obvious that the motility meter suffered from some serious deficiencies in the measurement of motor activity. One, the meter measures both exploratory and stereotyped locomotor activity (see section 2.16). Two, the meter will measure some but not all non-locomotor stereotyped behaviors, i.e., highly repetitive grooming, licking and sniffing movements. Three, the meter is not of adequate size to measure activity in rats more than 150 g in weight or for more than two rats. In order to circumvent these problems, the open-field (Gupta *et al.*, 1971; Gupta and Holland, 1972) was used as an alternative technique to measure activity. For rats the open-field was a 3' x 3' x 1' box with a formica floor divided into 9 squares using plastic electrical tape. Since it is essential that the open-field be thoroughly cleaned after each trial, the floor and sides must be constructed from waterproof material. Three feet above the floor of the open-field a 60 watt light bulb was positioned. By running the experiments in a dimly lit room, the light bulb prevented the animal from seeing the experimenter. The open-field used for mice was similar to that used for the rats except of a smaller size (1' x 1' x 1').

The essence of the usefulness of the open-field technique is that it presents the animal with a novel environment which it will explore. A normal and drug-treated animal was removed from his home cage and placed in the middle of the open field. The animal was then observed for a 5 to 10 min trial. Exploratory activity was registered as one count for every square completely crossed and one count for every rearing movement. Rearing behavior as an exploratory activity has been discussed by Gupta and Holland (1972). Stereotyped activity was measured as the total time the animals were engaged in highly repetitive non-locomotor behaviors. These behaviors in general consisted of sniffing, licking and other grooming movements.

In some experiments the motility meter was used as a two-square open-field by removing all photocells except for a single medial row of five cells. Stereotyped behavior was assessed when this arrangement of photocells were used as for the normal open-field.

## 3.13 CELL FREE *In vitro* PROTEIN SYNTHESIS.

Brain tissue was removed and homogenized in 4 volumes of 0.25 M sucrose, 12 mM $MgCl_2$, 100 mM KCl, 50 mM Tris·HCl, pH 7.6 (Medium I). Mitochondria and nuclear debris were sedimented by centrifugation at 12,000 g for 20 min at -4° C. The microsomal and soluble fractions were obtained by centrifuging the postmitochondrial supernatant at 105,000 g at 0° C for 1 hr.

*In vitro* protein synthesis was measured exactly as described by Zomzely *et al.* (1968). Approximately 2 mg of microsomal and 1 mg of soluble fraction protein were added to an incubation system containing 2 mM ATP (sodium salt), 0.25 mM GTP (sodium salt), 20 mM creatine phosphate (sodium salt), 0.1 mg creatine phosphokinase, Medium I, and 1 μCi of L-(U-$^{14}$C) leucine (specific activity, 250 mCi/mmol) in a final volume of 1 ml. Incubations were conducted at 37° C in open air. One-tenth milliliter aliquots removed from the incubation mixture at various times were precipitated in 1 ml of cold 5% TCA plus 1% unlabeled carrier leucine for 30 min at 0° C. The samples were then incubated at 90° C for 20 min and cooled at 0 to 4° C for an additional 15 min. The radiolabeled precipitate was collected onto filters as previously described.

## 3.14 SYNOPSIS OF CHAPTERS 4 TO 11.

The next chapter begins the first of seven chapters of experimental results. Due to the length of the material presented, it would seem prudent to provide the reader with summary statements of the major points developed in each chapter.

Chapter 4. In this chapter the effects of *d*-amphetamine and p-methoxyamphetamine (PMA) on DA and NE release and reuptake in tissue slices was studied. The data indicate that the reason PMA is a weak central stimulant and does not induce stereotype behaviors is because of the lack of effect the drug has on brain DA neurons. The major conclusion reached is that activation of both noradrenergic and dopaminergic neurons is required for the full expression of amphetamine's behavioral effects.

Chapter 5. The influence of *d*-amphetamine and the *d*-amphetamine metabolite and false transmitter p-hydroxynorephedrine (PONE) on *in vivo* and *in vitro* catecholamine synthesis, turnover and metabolism was studied. The accumulation of PONE in the brain is believed to be involved in the adverse behavioral effects resulting from chronic amphetamine administration. The data indicate that *d*-amphetamine in a dose

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related fashion first increases the release of newly synthesized DA and NE and, as the dose increases, inhibits catecholamine synthesis and metabolism. In general, 24 hr PONE pretreatment enhances the effect of amphetamine on NE release. Behaviorally, PONE pretreatment enhances the ability of challenging doses of *d*-amphetamine to induce stereotyped behaviors and decreases the ability of *d*-amphetamine to increase exploratory activity. It is concluded that PONE affects *d*-amphetamine behaviors through a direct receptor effect rather than an indirect effect on catecholamine synthesis.

Chapter 6. In Chapter 6 the effects of acute amphetamine intoxication on brain catecholamine synthesis and behavior were examined using the pellet implantation technique. The data indicate that after acute amphetamine intoxication the synthesis of brain DA is markedly increased. When the amphetamine pellet implanted animals are administered challenging doses of *d*-amphetamine at a time when there is no amphetamine remaining in the pellet (or in the animal's body), the predominant behavior produced is stereotyped activity. It is concluded that pellet implantation increases the DA to NE receptor activity ratio.

Chapter 7. The effect of chronic (2 weeks) amphetamine injections on *in vitro* catecholamine synthesis and behavior in the rat was examined in this chapter. Chronic amphetamine treatment changes the principal behavioral response caused by low to moderate challenging doses of amphetamine from increased exploratory activity to increased stereotyped activity. *In vitro* catecholamine synthesis was depressed but catecholamine release was increased in the chronically treated animals. Over time after withdrawal of amphetamine the effect on synthesis disappeared more rapidly than the effect on release. No significant effect on catecholamine reuptake was observed after chronic amphetamine treatment.

Chapter 8. The data in Chapter 7 suggested that chronic amphetamine treatment produces a semi-permanent increase in catecholamine release. The question asked in this and subsequent chapters is what membrane constituents are involved in this effect. Chapter 8 examines the role of various membrane constituents in NE release and reuptake. The data suggest that glycoproteins on the external surface of the nerve ending plasma membrane are intimately involved in NE uptake. Phospholipids, and especially the basic phospholipids, i.e., phosphatidylcholine and phosphatidylethanolamine, serve an intimate role in NE release. Decreasing membrane phospholipid content enhances NE release. With the experimental design used, phospholipids do not appear to serve a primary role in NE reuptake.

Chapter 9. Based on the findings in Chapters 7 and 8, in Chapter 9 the effect of acute and chronic amphetamine treatment on brain phosphatidylcholine synthesis and turnover was studied. The data demonstrate that amphetamine inhibits phosphatidylcholine synthesis and turnover. If decreased turnover can be equated with decreased functionality, then these data would support the hypothesis that the decrease in phosphatidylcholine turnover may be causally related to the chronic amphetamine-induced increase in NE (and perhaps DA) release.

Chapter 10. In Chapter 9 the effect of chronic amphetamine treatment on phosphatidylcholine turnover was examined in the major subcellular fractions, i.e., the mitochondrial, soluble and microsomal fractions. The important locus of the amphetamine effect, however, is the synaptic plasma membrane (SPM). The data in Chapter 10 show that chronic amphetamine treatment does not decrease, and may even increase, SPM phosphatidylcholine turnover. The SPM were derived from two populations of nerve endings, one of which is enriched in NE. The data in this chapter tend to discount the hypothesis developed in Chapter 9.

Chapter 11. In this chapter the effect of amphetamine on *in vivo* and *in vitro* protein synthesis was studied. Overall, the data demonstrate that amphetamine inhibits brain protein synthesis. The *in vitro* studies suggest that amphetamine interferes with one of the soluble enzymes involved in brain protein synthesis.

## CHAPTER 4

## A COMPARISON OF THE EFFECTS OF *dl*-PMA AND *d*-AMPHETAMINE ON CATECHOLAMINE RELEASE AND REUPTAKE AND ON BEHAVIOR.

### 4.1 INTRODUCTION.

After examining the literature on the acute mechanism(s) of *d*-amphetamine action (Section 2.13 to 2.16), it appeared that there were at least two areas in which further investigation was necessary before proceeding to an examination of the chronic drug effects. These two areas were (a) the relationship between *d*-amphetamine-induced locomotor stimulation and the activation of noradrenergic and dopaminergic neurons and (b) the effect of *d*-amphetamine on catecholamine synthesis. In this chapter some experiments designed to further elucidate topic (a) are described.

In a previous study it was found that *dl*-paramethoxyamphetamine (PMA) was equipotent to *d*-amphetamine *in vivo* in releasing $^{14}$C-DA and $^{14}$C-NE formed from $^{14}$C-tyrosine in the whole mouse brain (Hitzemann *et al.*, 1971). PMA was less potent than *d*-amphetamine in reducing endogenous DA and NE levels but more potent than *d*-amphetamine in increasing endogenous serotonin (5-HT) and decreasing endogenous 5-hydroxyindoleacetic acid (5-HIAA) levels. Although PMA had similar effects on *d*-amphetamine in the whole brain, it was observed that PMA did not induce stereotype behaviors or cause pronounced locomotor stimulation in doses up to 30 mg/kg i.p. Based on these data, it was decided to investigate the effects of PMA on catecholamine release and reuptake on a regional basis in hope of determining the reason(s) underlying the apparent paradoxical effects of PMA on catecholamines and on locomotor behavior. Some clue to the solution of this dilemma comes from the fact that PMA does not induce stereotyped behaviors. These data would suggest that PMA does not activate the dopaminergic neurons of the neostriatum.

## 4.2 EXPERIMENTAL - CHEMICAL.

The techniques used for measuring catecholamine uptake and release in tissue slices are described in Sections 3.9 and 3.10. Briefly, the procedures involved preparing tissue slices (0.26 mm) from the cortex and caudate nucleus and incubating the slices in KRB buffer (Katz and Kopin, 1969) with various concentrations of $^3$H-NE, $^3$H-DA or $^3$H-tyrosine. For the release experiments, the slices were loaded with the appropriate $^3$H-compound, washed several times and then reincubated in fresh buffer. The appearance of $^3$H-catecholamines in the buffer and the amount of residual $^3$H-catecholamine in the tissue were determined at various times. Column chromatography was used to purify the $^3$H-catecholamines in the tissue and medium from any metabolites that were formed. In some experiments a superfusion technique was employed for the release studies.

The initial rapid uptake of $^3$H-NE into the cortex and $^3$H-DA into the caudate nucleus was determined by incubating the slices prepared from these regions with various concentrations of $^3$H-NE or $^3$H-DA for 5 min (Snyder *et al.*, 1970). Uptake data were analyzed by Lineweaver-Burke plots and calculated as described by Wilkinson (1961).

## 4.3 EXPERIMENTAL - BEHAVIORAL.

Motor activity was measured using the Pharmacia MP 40/Fc motility meter using the photocell configuration which minimized counting from stereotyped locomotor and non-locomotor movements (see Section 3.12).

## 4.4 EFFECTS OF *dl*-PMA AND *d*-AMPHETAMINE ON $^3$H-DA AND $^3$H-NE RELEASE.

Using the method of Axelrod (1954) for amphetamine determination, preliminary studies indicated that the administration of 1 to 10 mg/kg i.p. of PMA or *d*-amphetamine to rats resulted in maximal brain amine levels of 6 to 60 μM or approximately 1 to 10 μg/g wet brain weight. Beginning in this concentration range and subsequently extending the studies to lower and higher concentrations as well, the effects of PMA and *d*-amphetamine on $^3$H-NE and $^3$H-DA release were investigated. The cortex was chosen as the region to measure NE release (and reuptake) in contrast to other brain regions which have a much higher density of NE nerve endings primarily because of the excellent slices which can be obtained. The slices from each cortical hemisphere were mixed together and then transferred to the appropriate tubes in order to decrease the possibility that anterior-posterior differences in the distribution of NE nerve endings would interfere with the interpretation of the results. The corpus striatum was chosen as the region to measure $^3$H-DA release (and reuptake) since this region is the principal locus of DA nerve endings in the brain (Glowinski and Baldessarini, 1966; Hornykiewicz, 1966). It should be noted that slices from the striatum were of poorer quality than those from the cortex in that they tended to break apart more readily which caused a greater variation in the total slice weight per incubation.

The data in Fig. 13a and 13b show the concentrations of PMA and *d*-amphetamine which had the maximum effect on $^3$H-NE and $^3$H-DA release. Some additional concentrations which were examined for a statistically appropriate number of times are shown as well. Six μM PMA or *d*-

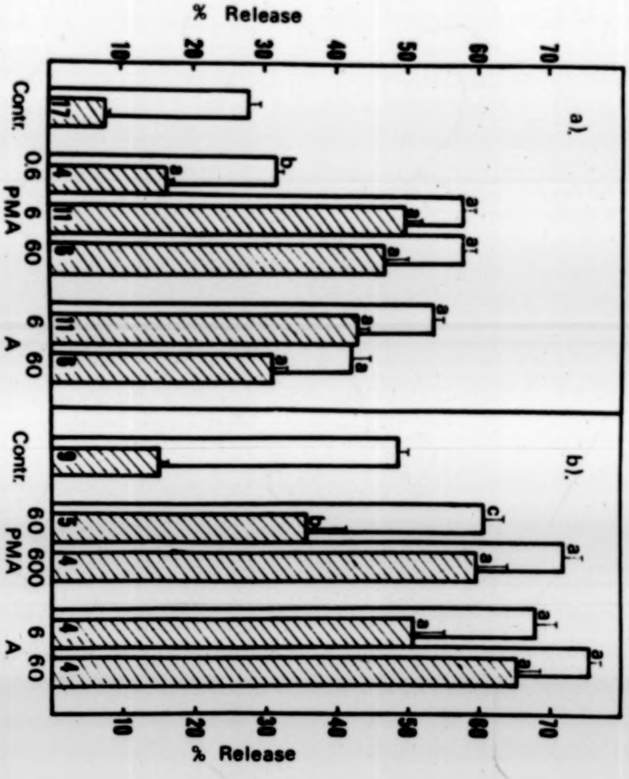
amphetamine was found to be the maximally effective dose to increase the release of $^3$H-NE and total $^3$H; both drugs appeared to be equally effective in increasing release (Fig. 13a). Higher doses of either drug did not result in a further increase in release and in the case of 60 μM *d*-amphetamine, a decrease in release from the previous drug dose was observed.

Differently than the data on $^3$H-NE release, PMA was found to be approximately 1/10 as potent as *d*-amphetamine in altering the release of total $^3$H and $^3$H-DA from slices prepared from the corpus striatum (Fig. 13b).

## 4.5 EFFECT OF *dl*-PMA AND *d*-AMPHETAMINE ON THE RELEASE OF $^3$H-DA FORMED FROM $^3$H-TYROSINE.

Although PMA was found to be ineffective in releasing $^3$H-DA in the corpus striatum taken up from the medium, based on the work of others (Weissman *et al.*, 1966; Sulser *et al.*, 1968; Hanson, 1967; Randrup and Munkvad, 1970) it may be more important to know the effect of PMA on DA which is synthesized in this brain region. This point seems important for two reasons. (a) It is not possible to know if the exogenous $^3$H-DA taken up from the medium actually equilibrates with all the DA stores, such that the $^3$H-DA will mimic the drug effects on endogenous DA; those arguments (Section 2.12) leveled against the intraventricular injection technique as a method to measure release *in vivo* can in part be applied to *in vitro* studies. (b) There is some evidence that amphetamine primarily acts on a small functional pool of newly synthesized catecholamines. The rightness or wrongeness of this concept is not to be examined here, but has been discussed previously (Section 2.15). Regardless of whether or not one is disposed to newly

Fig. 13. Effect of PMA and *d*-amphetamine on the release of $^3$H-NE from slices of the cerebral cortex and on the release of $^3$H-DA from slices of the corpus striatum. Slices (0.26 mm) of the cerebral cortex were preincubated with $^3$H-NE ($1 \times 10^{-7}$ M) and slices of the corpus striatum were preincubated with $^3$H-DA ($1 \times 10^{-6}$ M). After suitable washing procedures, the effect of PMA and *d*-amphetamine on the release of total $^3$H (open bar) and $^3$H-NE or $^3$H-DA (hatched bar) into fresh medium was measured. The data in Fig. 13a are from the cerebral cortex and those in Fig. 13b are from the corpus striatum. The numbers in the bars indicate the number of experiments. The numbers below the bars show the concentrations (μM) of PMA and *d*-amphetamine used. All data are expressed as the percent of total $^3$H in the tissue and medium released into the medium. a - $p < 0.001$; b - $p < 0.01$ and c - $p < 0.05$ as compared with control.


% Release
10
20
30
40
50
60
70
a)
b)
Contr.
0.6
6
60
PMA
6
60
A
Contr.
60
600
PMA
6
60
A
17
4
11
8
11
8
9
5
4
4
4
a
b
c
% Release
10
20
30
40
50
60
70

synthesized amine concept, the data in Fig. 14 illustrate that PMA was less effective than *d*-amphetamine to increase the release of newly synthesized $^3$H-DA. The total amount of $^3$H-DA found in the tissue and medium after PMA or *d*-amphetamine was not significantly different from control.

## 4.6 EFFECT OF *dℓ*-PMA AND *d*-AMPHETAMINE ON $^3$H-NE AND $^3$H-DA UPTAKE.

PMA (2.2 μM) was found to competitively block $^3$H-NE uptake into the cerebral cortex (Fig. 15). The same concentration of PMA was, however, only slightly effective in blocking $^3$H-DA uptake in the corpus striatum. A comparison of the $ID_{50}$ values for *d*-amphetamine and PMA indicates that PMA is 29 percent as effective as *d*-amphetamine in blocking uptake of $^3$H-NE in the cerebral cortex and only 14 percent as effective to block uptake of $^3$H-DA in the corpus striatum (Table 6).

## 4.7 EFFECT OF *dℓ*-PMA AND *d*-AMPHETAMINE ON $^3$H-DA AND $^3$H-NE RELEASE IN SUPERFUSED SLICES.

The experiments previously described cannot eliminate the possibility that the PMA and *d*-amphetamine induced increase in $^3$H-catecholamine release is not merely a reflection of inhibition of uptake. To examine this possibility, superfusion experiments which will minimize the contribution of uptake inhibition on release were performed. The concept that inhibition of uptake will not alter release in superfused slices was supported by a preliminary observation that desmethylimipramine (DMI) had no effect on $^3$H-NE release in cortical slices. The release of $^3$H-NE or $^3$H-DA from superfused tissue declined rapidly during the initial period of perfusion and became constant (Fig. 16). PMA or *d*-amphetamine in a concentration of 60 μM added to the super-

Fig. 14. Effect of PMA and *d*-amphetamine on the release of $^3$H-DA formed from $^3$H-tyrosine in the corpus striatum. Slices from the corpus striatum were incubated in the presence of 1 x $10^{-7}$ M $^3$H-tyrosine for 30 min, washed and reincubated for 20 min in the presence of PMA or *d*-amphetamine. All data is expressed as the precent of total $^3$H-DA in the tissue plus medium released into the medium. The numbers below the bars represent the concentration (μM) of PMA or *d*-amphetamine in the medium. a - $p < 0.001$; b - $p < 0.01$ compared to the control; *$p < 0.001$, **$p < 0.02$ compared to PMA.

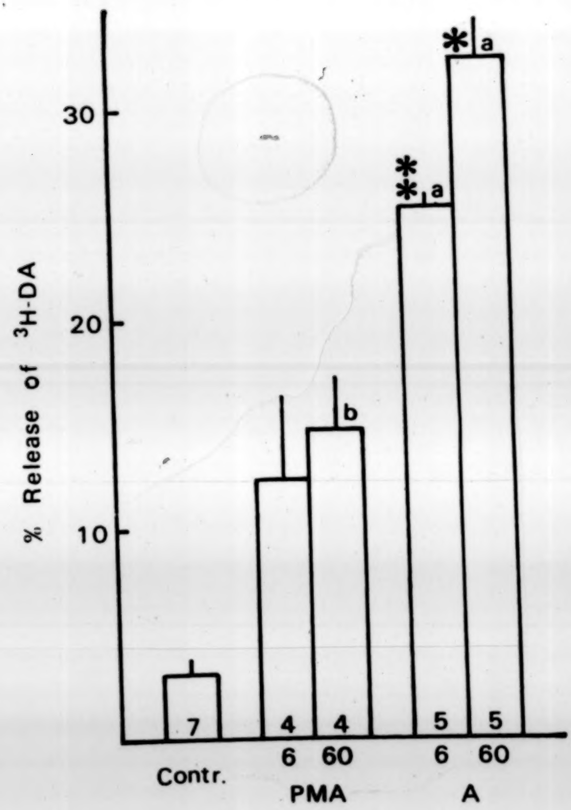


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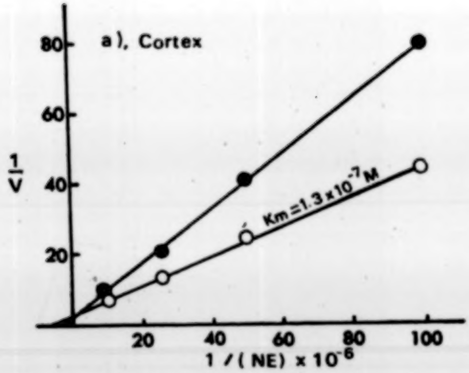
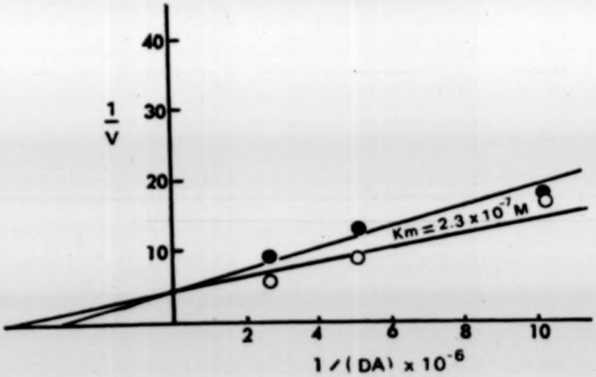
Fig. 15. Effect of PMA on the uptake of $^3$H-NE in the cerebral cortex and of $^3$H-DA in the corpus striatum. Slices prepared from the cortex and corpus striatum of the rat brain were incubated in the presence of varying concentrations of $^3$H-NE and $^3$H-DA and 22 μM PMA for 5 min. The reaction was terminated by rapidly washing the slices in an ice-cold medium. Lineweaver-Burke plots were prepared as described by Wilkinson (1961). Amine uptake (V) is given as mμmol/g/5 min. N = 3-4 experiments per point performed in duplicate. O - Control; ● - PMA.



a), Cortex

b). Corpus striatum



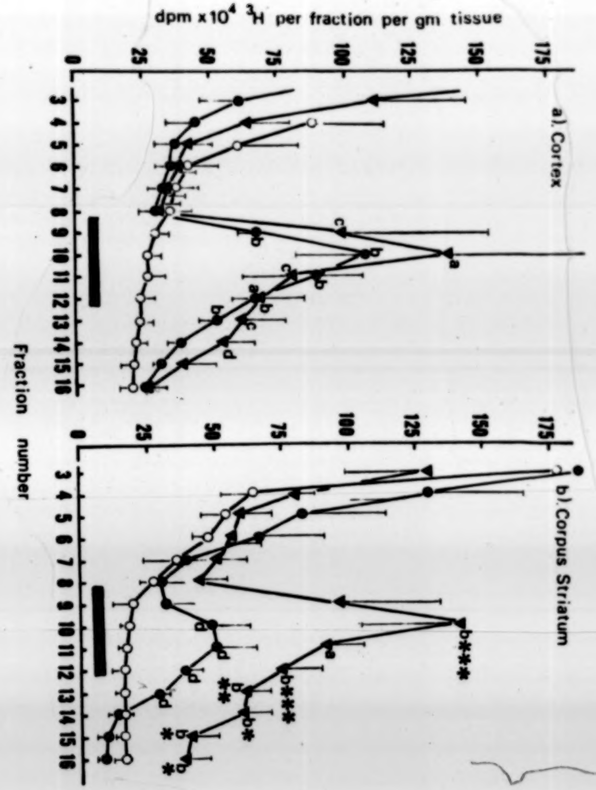
## TABLE 6

### Effects of *d*-Amphetamine and PMA on $^3$H-NE and $^3$H-DA Uptake [a]

| Group | ID$_{50}$<br>*d*-Amphetamine | PMA |
|---|---|---|
| $^3$H-NE-Cortex | $2.5 \times 10^{-6}$ | $8.6 \times 10^{-6}$ |
| $^3$H-DA-Corpus Striatum | $3.1 \times 10^{-6}$ | $2.2 \times 10^{-5}$ |

[a] Brain slices from the cerebral cortex and corpus striatum were incubated with $5 \times 10^{-7}$ M $^3$H-NE and $6 \times 10^{-7}$ M $^3$H-DA, respectively, in the presence of various concentrations ($10^{-7}$ - $10^{-5}$ M) *d*-amphetamine or PMA. The ID$_{50}$ values were determined by graphical analysis and each value represents the mean of three experiments.

Fig. 16. Effect of PMA and *d*-amphetamine on the release of $^3$H-NE from cortex slices (a) and of $^3$H-DA from striatal slices (b). Slices from the cortex and the corpus striatum were incubated in 1 x $10^{-7}$ M $^3$H-NE (cortex) or 1 x $10^{-6}$ M $^3$H-DA (corpus striatum), before washout and exposure to PMA or *d*-amphetamine in a superfusion apparatus. Each fraction represents 5 ml of the superfusion fluid. The horizontal black bar indicates the period in which PMA (60 μM) or *d*-amphetamine (60 μM) were added to the KRB buffer (9th to 12th fraction). Each point represents the mean ± S.E. of 5-8 experiments. All data is expressed as (dpm/fraction/gm tissue) x $10^{-4}$. a - $p < 0.001$; b - $p < 0.01$, c - $p < 0.02$; and d - $p < 0.05$ as compared with control; $^{*}p < 0.001$; $^{**}p < 0.01$; and $^{***}p < 0.05$ as compared with PMA. O - control; ● - PMA, ▼ - *d*-amphetamine.


a). Cortex
b). Corpus Striatum
dpm x10⁴ ³H per fraction per gm tissue
0
25
50
75
100
125
150
175
3 4 5 6 7 8 9 10 11 12 13 14 15 16
Fraction number
a
b
c
d
b***
b**
b*
**
*

fusion medium during the constant phase of release increased the release of $^{3}$H-NE in the cerebral cortex with little difference observed between the two drugs (Fig. 16a). However, in the corpus striatum, PMA was less effective than *d*-amphetamine to increase the release of $^{3}$H-DA (Fig. 16b). During the constant phase of release, the material released into the superfusion medium was found to be 90 percent or more $^{3}$H-NE or $^{3}$H-DA as determined by column chromatography.

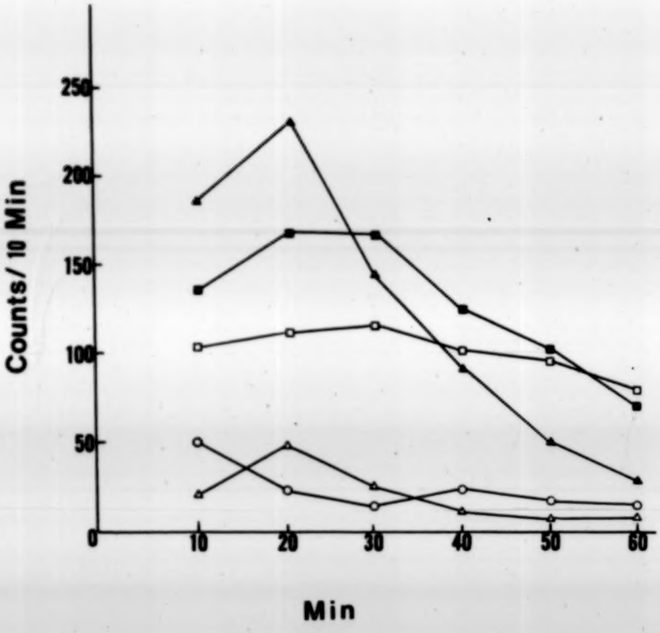
### 4.8 EFFECT OF *dℓ*-PMA AND *d*-AMPHETAMINE ON LOCOMOTOR ACTIVITY.

The effects of various doses of PMA and *d*-amphetamine on locomotor activity are shown in Fig. 17. The data confirm our previous findings in the mouse (Hitzemann *et al.*, 1971) that PMA is a weaker stimulant than *d*-amphetamine. In studies subsequent to those presented here, Tseng and Loh (1974) have demonstrated that the combination of PMA and apomorphine, a specific DA "receptor" stimulant, causes pronounced psychomotor stimulation.

### 4.9 DISCUSSION.

At one time the relative roles of NE and DA in *d*-amphetamine induced locomotor stimulation and stereotyped behaviors seemed quite clear (Randrup and Scheel-Krüger, 1966). While the development of stereotyped behaviors still appears to be primarily a neostriatal DA response (Randrup and Munkvad, 1970), recent evidence suggests that locomotor activity may result from a complex interaction between stimulated NE and DA receptors (Fuxe and Ugerstedt, 1970). As previously discussed, it is important to realize that there are at least two general patterns of locomotor activity exhibited by mice and rats, exploratory and stereotyped. The latter type, like non-locomotor stereotyped

Fig. 17. Effect of PMA and *d*-amphetamine on locomotor activity in the rat. Rats were administered saline or various doses of *d*-amphetamine or PMA i.p. and motor activity was measured for 1 hr on the Pharmacia MP/40 Fc motility meter. The motility meter was used in the photocell configuration which minimized counting of stereotyped movements. Data are from at least four rats per drug dose. 0 - control; Δ - PMA, 3 mg/kg; ▲ - PMA, 10 mg/kg; □ - *d*-amphetamine, 1 mg/kg; ■ - *d*-amphetamine, 3 mg/kg.


Counts/ 10 Min
250
200
150
100
50
0
10
20
30
40
50
60
Min

behaviors, is probably in part related to over-activation of DA "receptors". However, the question as to what degree DA "receptor" activation is involved in exploratory locomotor activity is unanswered. The evidence presented in this section would suggest that at least some DA "receptor" activation is required for the amphetamine-induced stimulation of exploratory locomotor activities.

PMA was found to be equipotent to *d*-amphetamine to increase NE release but was less potent to increase DA release. Although PMA is less potent than *d*-amphetamine to block the uptake of both NE and DA, the inhibition of uptake caused by PMA is apparently sufficient to block the metabolism of the released catecholamines as evidenced by the decreasing difference between the total $^3$H released and $^3$H-catecholamine released (Fig. 13a and 13b). Details of the mechanism by which uptake inhibition affects metabolism are given by Rutledge (1970). Overall, we can conclude that PMA is less effective than *d*-amphetamine to increase the extraneuronal DA concentration in the region of the synaptic cleft. The finding that a small amount of apomorphine given in combination with PMA produces pronounced locomotor stimulation (Tseng and Loh, 1974) supports the hypothesis developing from the present biochemical studies that activation of DA neurons involved in the mechanism by which *d*-amphetamine enhances exploratory locomotor activities.

On the basis of studies by Fuxe and Ugerstedt (1970) and data presented here, it may be convenient to picture the dose related effects of *d*-amphetamine on locomotor and stereotyped behaviors in the following manner. Arbitrarily assign an activity number of 1 to the DA and NE neurons affected by *d*-amphetamine. Behavioral studies correlated with chemical and anatomical data indicate that the mechanism by which low (0.5 mg/kg) doses of *d*-amphetamine facilitate behaviors such as the rate of conditioned avoidance responding is primarily related to activation of NE neurons (Wise and Stein, 1970). Thus, after a low dose of *d*-amphetamine the NE activity number may be raised to 2 while the DA activity number is still 1. When the dose of *d*-amphetamine is raised to 1 mg/kg i.p., hyperactivity mainly seen as increased exploratory locomotor stimulation results. At this dose level, the NE activity number probably increases to 3 or 4 but the DA activity number is also increased, for example to 2. As the dose of *d*-amphetamine is increased further, it is primarily the DA activity number which changes. At 3 to 6 mg/kg, the DA activity number may be 4 and the behavior observed is primarily stereotyped locomotor activities. When the DA activity number is increased still further by even larger doses of *d*-amphetamine, the principal behavioral response is non-locomotor stereotyped behaviors.

Such a conceptualization of the mechanism by which amphetamine alters behavior at different dose levels is clearly an oversimplification. The proposed hypothesis ignores the effects of amphetamine and PMA on other putative transmitters, e.g., ACh, GABA or 5-HT. The role of 5-HT may be particularly important. As indicated in the introductory section to this chapter, PMA markedly influences the levels of brain 5-HT and 5-HIAA. The possibility that these changes in 5-HT metabolism and the accompanying neurophysiological changes block the ability of PMA to enhance locomotor activity must be considered. Previously, it was demonstrated that pretreatment with p-chlorophenylalanine (PCPA), a serotonin depleting agent, potentiates the locomotor stimulatory effects of *d*-amphetamine (Hitzemann, 1970). These data indicate that the brain serotonin system does indeed have some influence and role in amphetamine-induced behaviors. Presently, the role of 5-HT in PMA and *d*-amphetamine induced behaviors is being investigated by Dr. Tseng in Dr. Loh's laboratory. In conclusion, in this and the succeeding chapters I will solely examine the role of the brain catecholamines in amphetamine-induced behaviors; the possibility, though, that other neurotransmitters are involved, especially in the chronic drug effects on behavior, must always be considered.

# CHAPTER 5

# EFFECTS OF *d*-AMPHETAMINE, PONE, AND PONE PLUS *d*-AMPHETAMINE ON CATECHOLAMINE SYNTHESIS, TURNOVER AND ON BEHAVIOR.

## 5.1 INTRODUCTION.

In the experiments described in this chapter, two separate but interrelated problems were examined simultaneously. The questions asked were (a) what is the effect of *d*-amphetamine on NE synthesis, and (b) what is the effect of PONE and PONE plus *d*-amphetamine on NE synthesis? Obviously, (a) is the control for (b) and, as a consequence, the data have been reported together.

As discussed in Chapter 2, various lines of evidence indicate that *d*-amphetamine preferentially increases the release of newly synthesized catecholamines (Weissman *et al.*, 1966; Sulser *et al.*, 1968; Hanson, 1967). This conclusion was primarily reached by observing that pretreatment with α-MT could block the locomotor stimulation and stereotyped activities induced by low to moderate doses of *d*-amphetamine. However, it must be remembered that α-MT has other effects on the brain catecholamines in addition to inhibition of tyrosine hydroxylase (Doteuchi *et al.*, 1974).

Based on the α-MT data, it was logical to proceed to analyze *in vivo* the effects of *d*-amphetamine on the formation of NE or DA from labeled tyrosine. The data obtained (Lewander, 1970; Costa and Groppetti, 1970; Glowinski, 1970; Beeson *et al.*, 1969, 1971; Javoy *et al.*, 1968; Persson, 1970) have been contradictory and, consequently, have led to some confusion concerning the effect of *d*-amphetamine. It is important to realize that the different results obtained by these authors may have been caused by differences in the methodology employed, e.g., differences in drug dose, route of precursor injection and the interval between drug and precursor administration. It may be that if a constant methodology were employed, it would be possible to demonstrate agreement between those studies which have measured the effect of amphetamine on the initial synthesis of catecholamine (e.g., Costa and Groppetti, 1970) and those studies which have measured the effect of *d*-amphetamine on the turnover or disappearance of newly synthesized catecholamines (Lewander, 1970). In the present experiments the effects of *d*-amphetamine on NE turnover and the *in vivo* and *in vitro* synthesis of NE were examined using a consistent experimental design.

It has been suggested that the accumulation of the *d*-amphetamine metabolite (Goldstein and Anagnoste, 1965) and false transmitter (Kopin *et al.*, 1965; Fischer *et al.*, 1965) p-hydroxynorephedrine (PONE) in noradrenergic nerve terminals may be responsible for the development of tolerance to some peripheral effects of *d*-amphetamine in rats and other species including man (Brodie *et al.*, 1969; Cavanaugh *et al.*, 1970). PONE is a weak sympathomimetic (Brodie *et al.*, 1969) which can be released by electrical stimulation ((Fischer *et al.*, 1965), nerve activity (Gill *et al.*, 1967), as well as by successive doses of *d*-amphetamine (Brodie *et al.*, 1970; Costa and Groppetti, 1970). Presumably, PONE displaces endogenous norepinephrine (NE) and competes with the residual NE for "receptor" sites. The effect of PONE on centrally *d*-amphetamine induced behaviors is not clear. Costa and Groppetti (1970) have suggested that the accumulation of PONE in the brains of chronic amphetamine abusers "may be important in producing their depression and psychotic reactions". More recently, Taylor and Sulser (1973) have observed that when fairly large doses of PONE or *d*-amphetamine are administered intraventricularly, the animals (rats) exhibited stereotyped behavior. PONE was found to deplete brain NE, increase the formation of $^3$H-normetanephrine from a pulse label of $^3$H-NE and decrease the formation of $^3$H-NE deaminated catechol metabolites.

From some of the reports cited above, it can be postulated that the accumulation of PONE in central noradrenergic nerve terminals would directly decrease the effectiveness of successive doses of *d*-amphetamine to induce those central behaviors such as increased exploratory activity which are primarily dependent on brain NE (see references in Fuxe and Ugerstedt, 1970, and conclusions in Chapter 4). Presumably, PONE would be released by *d*-amphetamine and compete with NE for binding to the receptor sites. However, Kopin *et al.* (1968) have demonstrated that false transmitters produce a persistent decrease in NE synthesis in the rat heart, an effect which would indirectly decrease NE mediated activities. The possibility that PONE may have an effect on brain NE similar to that described by Kopin *et al.* (1968) in the heart prompted an investigation of the effect(s) of PONE on brain NE synthesis and turnover. Based on the data of others that the accumulation of PONE may play an important role in the adverse effects caused by chronic amphetamine intoxication, it seems necessary to establish exactly what effects PONE has on NE metabolism independent of changes induced by *d*-amphetamine per se. Using the same reasoning, the effect of PONE on exploratory locomotor behavior was examined.

## 5.2 EXPERIMENTAL - CHEMICAL.

Sprague-Dawley rats (Simonsen Laboratories, Gilroy, California) weighing 180-200 g were used in pairs for all experiments. All animals were administered either vehicle or 10 μg of PONE intracisternally (i.cist.) in 0.9 percent saline 24 hr prior to behavioral or chemical studies. For the *in vivo* $^3$H-catecholamine experiments, animals were administered either saline (0.5 ml) or *d*-amphetamine sulfate (1-3 mg/kg, as base) i.p. 0.5 hr prior to the administration of 20 Ci (0.35 μg) of 2',3'-$^3$H-tyrosine i.cist. At various times, the animals were sacrificed by decapitation and the brains rapidly removed. The cortex and the cerebellum were dissected away and the remainder of the brain, the diencephalon-midbrain-brain stem (DMB), was homogenized in 5 volumes of 0.4 N $HClO_4$. In some experiments animals were administered the $^3$H-tyrosine 1 hr prior to drug administration. In these experiments, animals were sacrificed at 0, 30 and 60 min after drug administration.

For the *in vitro* NE synthesis experiments, animals were administered *d*-amphetamine in saline 0.5 hr prior to sacrifice. Slices were prepared from the cortex and brain stem (pons-medulla) and *in vitro* NE synthesis was determined as described by Harris and Roth (1971). Further details of the *in vitro* technique and of the isolation of $^3$H-NE and $^3$H-NE metabolites in both the *in vivo* and *in vitro* studies are given in Sections 3.8 and 3.11.

## 5.3 EXPERIMENTAL - BEHAVIORAL.

Since it was especially crucial to the understanding of the effects of PONE on locomotor behavior that the behavior measured was exploratory locomotor activity (a primarily NE related phenomenon, activity was measured using the open-field apparatus (Gupta *et al.*, 1971; Gupta and Holland, 1972, see Section 3.12). One hr after *d*-amphetamine or saline administration, individual animals were placed in the large open field for a single 5 min trial and exploratory and stereotyped behavior was measured.

## 5.4 RESULTS - INTRODUCTORY COMMENTS.

PONE pretreatment consisted of a single i.cist. injection of 10 µg 24 hr prior to behavioral or chemical studies. This dose was chosen since it produced a depletion of NE similar to that caused by 10 mg/kg of *d*-amphetamine i.v. (Costa and Groppetti, 1970) and thus it would be possible to compare and relate our results with the detailed studies of Costa and Groppetti (1970).

The use of a 24 hr pretreatment with PONE was thought desirable for several reasons. PONE is normally formed from *d*-amphetamine via hepatic p-hydroxylation followed by transfer of the p-OH-amphetamine to sites containing DA-β-hydroxylase and subsequent metabolism by this enzyme. However, when substances are injected directly into the brain, the distribution of the injected compound is probably quite different than that observed when it is formed endogenously. This point is especially true when injecting PONE since the commercially available compound is a racemic mixture. It was thought that a 24 hr pretreatment would decrease the possibility that the changes observed were the result of excess extraneuronal PONE or from PONE taken up non-specifically. Hopefully, the PONE isomer which corresponds to the PONE formed endogenously would persist in the brain longer than the other isomers as a result of its storage in the NE granule and its slow release. However this point is not proven experimentally and to what degree the "non-physiological" isomers persist in the brain is unknown.

PONE was administered i.cist. despite the disadvantages of the ether anesthesia for the following reasons. As evidenced by the results of Taylor and Sulser (1973), when PONE is administered intraventricularly via a permanent cannulae, a substantial amount of the PONE is taken up into the DA neurons of the caudate nucleus. Since we were interested in examining the effects of PONE only on NE neurons, the i.cist. route was more desirable. The distribution of compounds injected i.cist. is shown in Chapter 9. Very little of the injected material reaches the caudate nucleus. Since PONE was given i.cist., the subsequent injections of $^3$H-tyrosine were also given by this route.

### 5.5 EFFECT OF PONE AND *d*-AMPHETAMINE ON OPEN-FIELD ACTIVITY.

The data in Fig. 18 show that in animals pretreated with PONE, the administration of *d*-amphetamine in doses ranging from 1 to 10 mg/kg decreased exploratory activity from control. Simultaneously, PONE enhanced the effectiveness of *d*-amphetamine to produce stereotyped behaviors. It should be noted that the level of stereotyped behaviors in the PONE control group was significantly greater than in the normal control.

### 5.6 EFFECT OF PONE AND *d*-AMPHETAMINE ON BRAIN NE LEVELS.

PONE pretreatment caused a 43 percent decrease in brain NE levels (Fig. 19). The administration of 1 or 3 mg/kg of *d*-amphetamine caused a further decrease in endogenous NE levels. By themselves, these doses of *d*-amphetamine had no effect on brain NE.

### 5.7 EFFECT OF PONE AND *d*-AMPHETAMINE ON THE FORMATION OF $^3$H-NE FROM $^3$H-TYROSINE.

Animals receiving various drug treatments were administered 20 μCi of $^3$H-tyrosine (specific activity 11.4 Ci/mmol) i.cist. The levels of $^3$H-NE and the specific activity of tyrosine were determined 5, 15 and

Fig. 18. Effect of PONE and *d*-amphetamine on exploratory and stereotyped behaviors. Saline and PONE 24 hr pretreated rats were administered various doses of *d*-amphetamine i.p. One hr later individual rats were given a single 5 min trial in the open field and scored for exploratory and stereotyped behaviors. Exploratory activity is expressed in counts/min (cpm) and stereotyped behavior is expressed in min. N = 8-12 animals per group. ☐- saline pretreated rats; ▣ - PONE pretreated rats. * - PONE group significantly different than the appropriate control. $p < 0.05$.

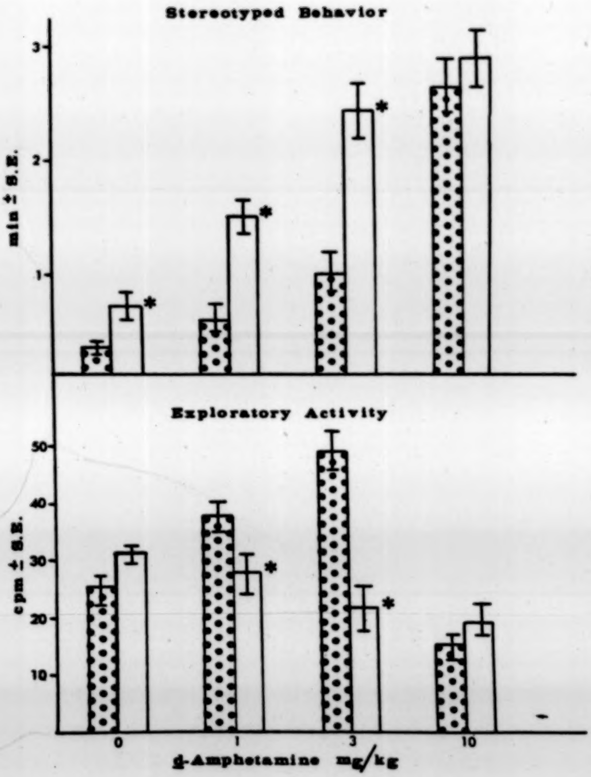
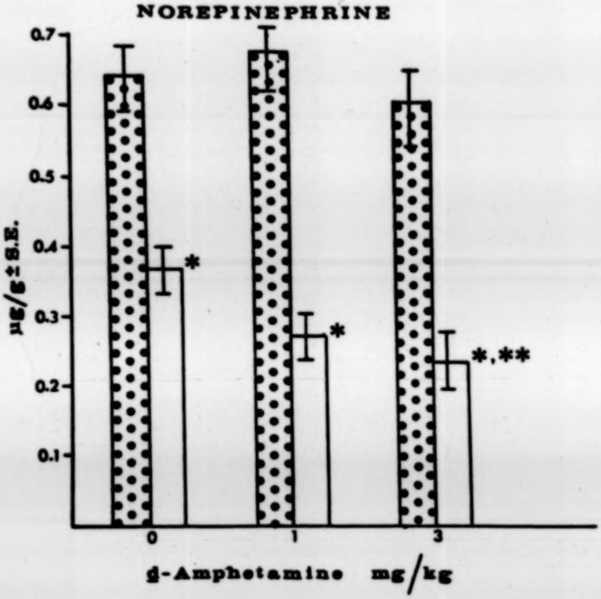

Stereotyped Behavior
min ± S.E.
3
2
1
*
*
*
Exploratory Activity
cpm ± S.E.
50
40
30
20
10
*
*
0
1
3
10
d-Amphetamine mg/kg

Fig. 19. Effect of PONE and *d*-amphetamine on brain norepinephrine levels. Rats were administered 10 μg of PONE or saline vehicle i.cist. 24 hr prior to the injection of various doses of *d*-amphetamine i.p. One hr after *d*-amphetamine, the levels of NE were determined in the diencephalon-midbrain-brain stem (DMB). All data are expressed as mean μg/g ± S.E. N = 6-8 determinations per group. ▣ - saline pretreated rats; □- PONE pretreated rats. * - PONE group significantly different than respective saline control. $p < 0.05$. ** - Significantly different than the PONE plus saline group. $p < 0.05$.


NOREPINEPHRINE
0.7
0.6
0.5
0.4
0.3
0.2
0.1
µg/g ± S.E.
*
*
*,**
0
1
3
d-Amphetamine mg/kg

30 min later. Preliminary experiments indicated that the peak accumulation of $^3$H-NE occurred 30 to 45 min after $^3$H-tyrosine administration. The data in Fig. 20 illustrate the effects of the various drug treatments on the formation of $^3$H-NE. All data is expressed as the quotient of $^3$H-NE levels divided by the specific activity of tyrosine (Azmitta *et al.*, 1970). In both the control and PONE pretreated groups, the administration of *d*-amphetamine significantly decreased the apparent formation of $^3$H-NE at 30 min. Significant changes were not observed at earlier time points. Chronic PONE treatment per se had no effect on apparent $^3$H-NE formation.

## 5.8 EFFECT OF PONE AND *d*-AMPHETAMINE ON THE TURNOVER OF $^3$H-NE.

Normal and PONE pretreated rats received 20 μCi of $^3$H-tyrosine 1 hr prior to the administration of *d*-amphetamine. Following *d*-amphetamine administration, the decay of $^3$H-NE levels was followed for 1 hr. During this first hour of turnover there was a rather rapid disappearance of $^3$H-NE. Preliminary experiments indicated in subsequent time intervals the rate of turnover was slower. While the phase of rapid turnover is convenient for examining acute drug effects, it would not be suitable for determining the true rate of $^3$H-NE disappearance. The data in Fig. 21 illustrate that in the control group *d*-amphetamine decreased the disappearance of $^3$H-NE in a dose related fashion. However, in the PONE pretreated group *d*-amphetamine enhanced $^3$H-NE disappearance. PONE pretreatment alone slightly enhanced $^3$H-NE disappearance. In no group were significant changes observed in the time course of disappearance for the specific activity of tyrosine.

Fig. 20. Effect of PONE and *d*-amphetamine on the formation of $^{3}$H-NE from $^{3}$H-tyrosine. Rats were administered PONE and *d*-amphetamine as described in the legend to Fig. 2. One-half hr after *d*-amphetamine, the animals were given 20 µCi of $^{3}$H-tyrosine i.cist. The animals were sacrificed at various times and the levels of $^{3}$H-NE, $^{3}$H-tyrosine and endogenous tyrosine were determined in the DMB. All data are expressed as the ratio of [$^{3}$H-NE (cpm/g)/ H-tyrosine (cpm/nmol)] ± S.E. N - 6-8 determinations per group. The upper graph shows the PONE pretreated rats and the lower graph the saline pretreated rats. ● - no amphetamine; 0 - 1 mg/kg amphetamine; ▲ - 3 mg/kg amphetamine; ●---● - no amphetamine control. * - Significantly different than the control value. $p < 0.05$.

XUM

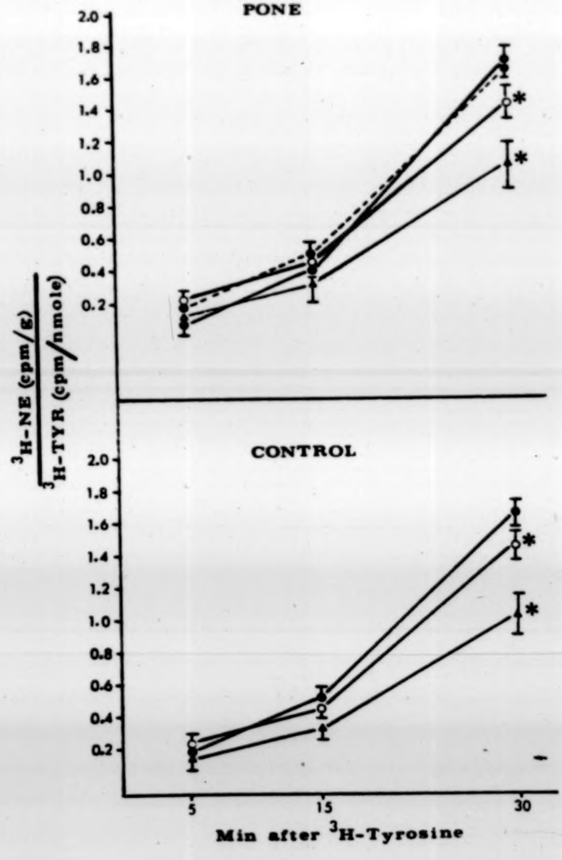

PONE
CONTROL
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
*
*
5
15
30
Min after ³H-Tyrosine
³H-NE (cpm/g) / ³H-TYR (cpm/nmole)

Fig. 21. Effect of PONE and *d*-amphetamine on the disappearance of $^{3}$H-NE. Rats were pretreated with 10 μg of PONE i.cist. 24 hr prior to the injection of 20 μCi of $^{3}$H-tyrosine i.cist. One hr later the animals were given various doses of *d*-amphetamine i.p. The animals were sacrificed at 0, 30 and 60 min after *d*-amphetamine and the specific activity of NE was determined in the DMB. All data are expressed as the log (cpm/nmol ± S.E.). N = 5-6 determinations per point. The upper graph shows the PONE pretreated animals and the lower graph the saline pretreated animals. ● - no amphetamine; ○ - 1 mg/kg amphetamine; ▲ - 3 mg/kg amphetamine; ●---● - no amphetamine control. * - Significantly different than the no amphetamine group. $p < 0.05$.

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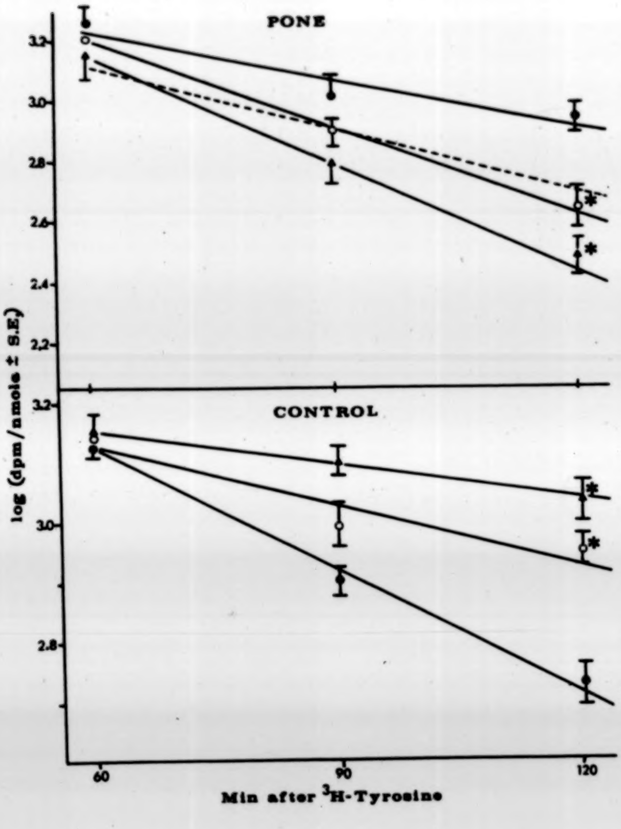
PONE
CONTROL
3.2
3.0
2.8
2.6
2.4
2.2
log (dpm/nmole ± S.E)
*
60
90
120
Min after $^3$H-Tyrosine

## 5.9 EFFECT OF PONE AND *d*-AMPHETAMINE ON THE TURNOVER OF $^3$H-MOPEG.

The formation of $^3$H-MOPEG was measured in the same manner as described for $^3$H-NE in the legend to Fig. 20. In the control group 1 mg/kg of *d*-amphetamine did not significantly alter $^3$H-MOPEG levels but 3 mg/kg significantly decreased $^3$H-MOPEG levels at 30 min (Fig. 22). PONE pretreatment markedly increased $^3$H-MOPEG formation and this effect was enhanced by *d*-amphetamine.

## 5.10 EFFECT OF PONE AND *d*-AMPHETAMINE ON THE "*In vitro*" FORMATION OF $^3$H-NE.

The effect of various drug treatments on $^3$H-NE formation in tissue slices was determined using the methodology of Harris and Roth (1971). *d*-Amphetamine enhanced $^3$H-NE release as evidenced by a decrease in the T/M ratio in both the brain stem and cortex but also decreased the net (tissue + medium) synthesis of $^3$H-NE in these brain regions (Table 7). Differently, PONE pretreatment enhanced $^3$H-NE release in both brain regions but also caused a small increase in the net synthesis of $^3$H-NE in the brain stem. In the PONE plus *d*-amphetamine treated group, the release of $^3$H-NE was further increased but no significant change occurred in the net synthesis of $^3$H-NE as compared to the PONE control in both brain regions.

## 5.11 EFFECT OF PONE AND *d*-AMPHETAMINE ON TYROSINE HYDROXYLASE ACTIVITY.

The data in Table 8 show that PONE pretreatment increased tyrosine hydroxylase activity. However, this increased enzyme activity could be reversed by *d*-amphetamine. *d*-Amphetamine alone had no effect on tyrosine hydroxylase activity.

Fig. 22. Effect of PONE and *d*-amphetamine on the formation of $^{3}$H-MOPEG. Rats were administered PONE, *d*-amphetamine and $^{3}$H-tyrosine as described in the legend to Fig. 3. At various times the animals were sacrificed and the levels of $^{3}$H-MOPEG in the DMB determined. All data expressed as (cpm/g ± S.E.) x $10^{-2}$. The upper graph shows the PONE pretreated group and the lower graph the saline pretreated group. ● - no amphetamine; 0 - 1 mg/kg amphetamine; ▲ - 3 mg/kg amphetamine; ●---● - no PONE control. * - Significantly different than control. $p < 0.05$.

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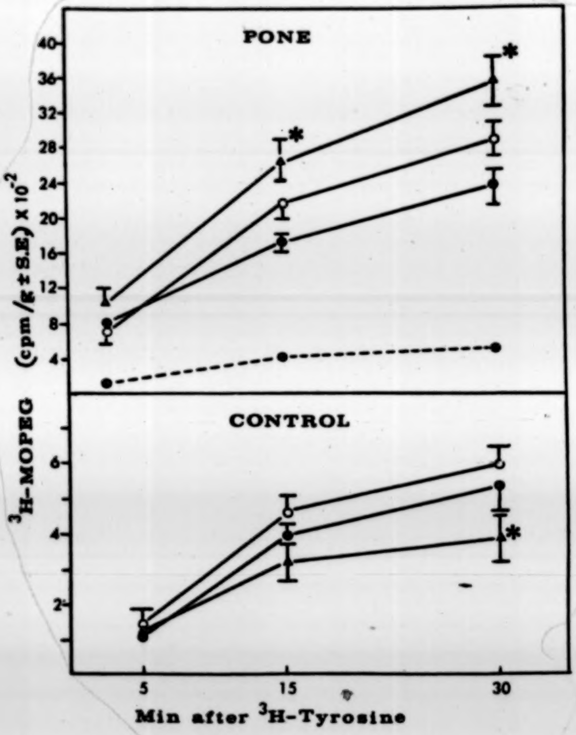
PONE
CONTROL
$^3$H-MOPEG (cpm/g ± S.E) x 10$^{-2}$
40
36
32
28
24
20
16
12
8
4
6
4
2
5
15
30
Min after $^3$H-Tyrosine
*
*
*

## TABLE 7

Effect of PONE and *d*-Amphetamine on the *in vitro* Synthesis of NE.[a]

|  | NE nmol/g/hr ± S.E. |  |  |  |  |  |
|---|---|---|---|---|---|---|
|  | Brain Stem |  |  | Cortex |  |  |
| Group | T | M | T/M | T | M | T/M |
| Control | 1.20 ± 0.11 | 0.10 ± 0.02 | 12.0 | 0.24 ± 0.02 | 0.02 ± 0.01 | 8.0 |
|  | (1.30 ± 0.10)[b] |  |  | (0.27 ± 0.02) |  |  |
| Amphetamine 3 mg/kg | 0.75 ± 0.08* | 0.13 ± 0.03 | 5.7 | 0.14 ± 0.02* | 0.04 ± 0.01 | 3.5 |
|  | (0.88 ± 0.09)* |  |  | (0.19 ± 0.02)* |  |  |
| PONE | 1.46 ± 0.12 | 0.23 ± 0.04* | 6.4 | 0.29 ± 0.03 | 0.06 ± 0.01* | 4.8 |
|  | (1.68 ± 0.11)* |  |  | (0.35 ± 0.04) |  |  |
| PONE + Amphetamine 3 mg/kg | 1.35 ± 0.13 | 0.39 ± 0.05*† | 3.3 | 0.24 ± 0.03 | 0.10 ± 0.01*† | 2.4 |
|  | (1.74 ± 0.13)* |  |  | (0.34 ± 0.04) |  |  |

*a* - Rats were administered PONE and *d*-amphetamine as described in the legend to Fig. 3. Thirty min after *d*-amphetamine the animals were sacrificed and tissue slices (0.26 mm thickness) were prepared from the brain stem and cortex. Slices were incubated in the presence of 2 x $10^{-5}$ M $^3$H-tyrosine for 30 min as described by Harris and Roth (1971). The rates of NE accumulation were determined in the tissue (T) and medium (M).

*b* - Data in parentheses are the net (tissue + medium) synthesis of NE.

* - Significantly different than control, $p < 0.05$.

† - Significantly different than the PONE control, $p < 0.05$.

## TABLE 8

### Effects of PONE and PONE plus *d*-Amphetamine on Cerebral Tyrosine Hydroxylase Activity [a]

| Group | Tyrosine Hydroxylase Activity (dpm/hr/mg protein) x $10^{-3}$ |
|---|---|
| Control | 16.1 |
| *d*-Amphetamine, 3 mg/kg | 17.4 |
| PONE | 25.3 |
| PONE + *d*-Amphetamine, 3 mg/kg | 18.7 |

[a] Animals were administered drugs as described in Table 3. Tyrosine hydroxylase activity was determined in the diencephalon-midbrain-brain stem 1 hr after *d*-amphetamine administration as described by McGeer *et al.* (1967). Data represent the mean of 4 determinations.

## 5.12 DISCUSSION OF THE EFFECTS OF *d*-AMPHETAMINE ON NE SYNTHESIS AND TURNOVER.

The study of the effects of *d*-amphetamine on the formation of labeled NE and DA from labeled tyrosine has led to somewhat conflicting results. Costa and Groppetti (1970) and Persson (1970) have reported that *d*-amphetamine preferentially increases the cerebral accumulation of $^{14}$C-DA from $^{14}$C-tyrosine in the whole brain and caudate nucleus. Beeson *et al.* (1969, 1971) have observed that *d*-amphetamine decreases $^{3}$H-DA formation from $^{3}$H-tyrosine in the caudate nucleus but increases $^{3}$H-NE formation in the brain stem. Lewander (1970) has found that *d*-amphetamine decreases the turnover of $^{3}$H-DA but not $^{3}$H-NE in the whole brain of animals pretreated with $^{3}$H-tyrosine. Recently Weiner *et al.* (1972) have observed that *d*-amphetamine decreases tyrosine hydroxylase activity in the intact mouse vasa deferentia.

Weiner *et al.* (1972) and Beeson *et al.* (1971) have suggested the following mechanism to account for the decrease in synthesis. Amphetamine will release catecholamines from storage granules and thus increase the intraneuronal level of "free" catecholamines. The increased levels of unbound catecholamines inhibit tyrosine hydroxylase by competing with the reduced pteridine cofactor for the oxidized enzyme (see Ikeda *et al.*, 1966). The present experiments provide supportive evidence for the view that *d*-amphetamine decreases catecholamine synthesis. The data shown in Fig. 20 indicate that *d*-amphetamine decreases the apparent formation of $^{3}$H-NE. In our opinion, this decrease in $^{3}$H-NE is likely to be related to a decrease in $^{3}$H-NE synthesis for the following reasons. (a) The results of the turnover experiments (Fig. 21) indicate that *d*-amphetamine decreases $^{3}$H-NE turnover. As described by Neff *et al.* (1971), a decrease in turnover implies a decrease in synthesis. (b) The *in vitro* studies show that *d*-amphetamine both increases the release and decreases the net (tissue + medium) synthesis of $^3$H-NE in the cortex and brain stem. Similar results were obtained by Beeson *et al.* (1971) when they measured the effect of *d*-amphetamine on the formation of $^3$H-DA formed from $^3$H-tyrosine in slices prepared from the caudate nucleus.

## 5.13 DISCUSSION OF THE EFFECTS OF PONE AND PONE PLUS *d*-AMPHETAMINE ON NE SYNTHESIS AND TURNOVER.

The present study suggests that chronic PONE treatment has at least the following effects on NE synthesis, storage and metabolism. (a) PONE increased the formation of $^3$H-MOPEG from $^3$H-tyrosine. However, this increase in $^3$H-NE metabolism did not result in a decrease in $^3$H-NE levels probably as a result of the increase in tyrosine hydroxylase activity. (b) After PONE pretreatment, endogenous NE is more easily released by *d*-amphetamine (Fig. 19). Apparently PONE not only displaces NE from storage sites, but also decreases the stability of the residual NE. (c) PONE pretreatment alone has no effect on $^3$H-NE disappearance (Fig. 21), but PONE plus *d*-amphetamine enhanced the disappearance of $^3$H-NE. In the PONE treated animals, the disappearance of $^3$H-NE cannot be equated with turnover since the endogenous levels of NE have changed. Thus, an increase in $^3$H-NE disappearance, in this case, does not imply an increase in synthesis. Rather, it would appear that the increase in $^3$H-NE disappearance is related to at least two factors. (a) PONE plus *d*-amphetamine caused a further increase in the formation of $^3$H-MOPEG. MOPEG is a major metabolite of NE in the rat brain (Schandberg *et al.*, 1968) and under certain conditions its formation can be used to measure NE turnover (Meek and Neff, 1972). Ideally, to determine if various drug treatments enhance NE metabolism, it would be prudent to measure several metabolites. In situations where *d*-amphetamine is used, it has in the past proven useful to examine changes in $^3$H-normetanephrine (NM) and the $^3$H-deaminated NE metabolites (Glowinski and Axelrod, 1965; Glowinski *et al.*, 1966; Taylor and Sulser, 1973). However, in our experience (see Section 3.9), only the levels of $^3$H-MOPEG (or $^3$H-homovanillic acid (HVA) can be accurately determined when $^3$H-tyrosine is used as the precursor. Although one cannot say with surety on the basis of changes in $^3$H-MOPEG levels alone that PONE or PONE plus *d*-amphetamine treatment has enhanced $^3$H-NE metabolism, there appears to be a good possibility enhanced metabolism occurs.

(b) The *in vitro* data suggest that in the PONE plus *d*-amphetamine treated animals there is enhanced release of the newly synthesized NE but this was not accompanied by a decrease in synthesis as observed with *d*-amphetamine alone. Kopin *et al.* (1968) have postulated that acute false transmitter treatment inhibits tyrosine hydroxylase indirectly by increasing the levels of free intraneuronal NE. In the chronically PONE treated animals we have observed what is probably one form of adaptation to this effect; namely, enhanced tyrosine hydroxylase activity. Other forms of adaptation may occur as well. In the present study it was found that PONE pretreatment tended to enhance $^3$H-NE synthesis *in vitro* in the brain stem which is perhaps a reflection of the increased tyrosine hydroxylase activity. It should be noted that this *in vitro* increase in $^3$H-NE formation differs from the *in vivo* data (Fig. 20) where no change in the formation of $^3$H-NE was observed. This difference may result from the fact that *in vitro* the formation of labeled NE metabolites when labeled tyrosine is used as the precursor substance occurs fairly slowly (Harris and Roth, 1971). Importantly, one aspect of the acute mechanism by which false transmitters inhibit NE synthesis is similar to that postulated for *d*-amphetamine-induced inhibition (Weiner *et al.*, 1972; Beeson *et al.*, 1971). Based on the *in vitro* studies, there appears to be some "cross-tolerance" between PONE and *d*-amphetamine with regard to the inhibition of NE synthesis. The fact that PONE pretreatment plus *d*-amphetamine causes the enhanced release of the newly synthesized NE and depletes endogenous NE stores (Fig. 19) without inhibition of synthesis may explain the observation that this drug treatment increases the disappearance of $^3$H-NE *in vivo* (Fig. 21).

Chronic PONE pretreatment was found to block the *d*-amphetamine-induced increase in exploratory activity and enhance the production of *d*-amphetamine-induced stereotyped behaviors (Fig. 18). Various studies have shown that the *d*-amphetamine-induced increase in exploratory activity is in part related to an increase in NE "receptor" activity (Fuxe and Ugerstedt, 1970; Randrup and Scheel-Krüger, 1966). However, from the foregoing discussion, it appears that PONE pretreatment tends to enhance the ability of *d*-amphetamine to increase the level of NE in the region of the receptor. At this time it is concluded that PONE alters the *d*-amphetamine induced behaviors by a mechanism not related to changes in NE synthesis and metabolism. Brodie *et al.* (1970) have suggested that PONE is preferentially released by subsequent doses of *d*-amphetamine and the competition of the weak sympathomimetic PONE with NE for available receptor sites decreases noradrenergic nerve activity.

## 5.14 GENERAL COMMENTS.

The results presented in this chapter suggest that many of the conflicting results obtained by others on the effect of *d*-amphetamine on brain catecholamine synthesis can be eliminated by using a consistent methodological and experimental approach. The effects of any drug, including amphetamine, on the brain catecholamines depend on a variety of factors including the experimental conditions, the strain and/or species of animal used, the drug dose used, the time course employed to examine the drug effects and, when labeled precursors are used, the route of precursor administration. Rarely is there an attempt to duplicate the results of others by using exactly the same experimental conditions. Often, though, this is the fault of the original investigator for not spelling out in detail the experimental conditions employed. When considering all these points, it is not surprising that vastly different results are obtained from one laboratory to the next when studying the brain catecholamines.

The fact that in the present study fairly similar results were obtained with both *in vivo* and *in vitro* methods indicates that the *in vitro* technique with its inherently greater reproducibility may be a valuable and powerful technique for studying the effects of drugs on the brain catecholamines and other putative transmitters. However, there are important points that must be realized when using the *in vitro* tissue slice technique. One, the tissue is rapidly dying, even under the most optimum conditions. Thus, speed in handling the tissue is of utmost importance. Two, the buffer used can influence the results obtained. In hindsight, it probably would have been useful to add additional energy sources, e.g., glutamate, lactate and pyruvate, to the incubation medium. Harris and Roth (1971) suggested that a phosphate buffer be used to measure catecholamine synthesis in slices and this buffer was used in the present study. However, when $CO_2$ is bubbled through a phosphate rather than a bicarbonate buffer, $CO_2$ fixation is much more likely to occur. Thus, I would recommend to anyone interested in using the *in vitro* that a bicarbonate buffer be employed. Three, as the slices become less viable during the course of the incubation, the electrical activity of the neurons also decreases. Ideally, the slices should be mildly stimulated. This would increase respiration and make the slices more nearly normal. However, stimulation would severely limit the number of incubations performed per experiment.

# CHAPTER 6

# ACUTE *d*-AMPHETAMINE INTOXICATION IN THE MOUSE - EFFECT OF PELLET IMPLANTATION

## 6.1 INTRODUCTION.

In the previous chapter one aspect of the relationship between catecholamines and behavior in amphetamine intoxication was investigated; namely, the effects of PONE. However, there are additional aspects which need also to be considered. The problem to be examined in this and the subsequent chapter is the determination of what effects amphetamine per se has in intoxication phenomena. Although some attempts to examine this problem in the rat are presented in Chapter 7, the problem is greatly simplified by using a species such as the mouse which does not accumulate amphetamine metabolites in the brain (Hitzemann, 1970).

In a previous study (Hitzemann, 1970) some preliminary investigations were made of a pellet implantation technique as a model for studying intoxication in the mouse. The formulation of the pellet was such that the drug was "leached" from the pellet at a fairly rapid rate; the half-life ($t_{\frac{1}{2}}$) of the pellet-amphetamine was approximately 4 hours. Despite the rapid release of the amphetamine from the pellet, it was observed that tolerance developed after a single pellet implantation to amphetamine's effect on exploratory activity. The mechanism(s) underlying this tolerance phenomena did not appear to involve an effect of amphetamine on catecholamine synthesis, storage or metabolism. In this chapter some additional chemical and behavioral effects of pellet implantation are presented. The continuation of the implantation experiments in the context of the present studies seemed indicated for at least two reasons. (a) The mouse is probably a better species than the rat in which to examine purely amphetamine effects since neither p-OH-amphetamine nor PONE accumulate in the mouse brain after amphetamine administration (Hitzemann, 1970). (b) The observation that tolerance developed to a locomotor effect of amphetamine is fairly unique in that most other investigators have not observed such a phenomenon (Brodie *et al.*, 1969; Lewander, 1971; Schuster *et al.*, 1961). The question arose as to whether or not pellet implantation has a unique effect on amphetamine-induced stereotyped behaviors? Does tolerance develop to these activities as well? Experiments designed to answer these questions should be performed.

## 6.2 EXPERIMENTAL - CHEMICAL.

Male CF-1 mice (Horton Laboratories, Oakland, Calif., or Carworth Farms, Portage, Mich.) weighing 20-25 g were used in all experiments. Mice to be implanted with amphetamine or placebo pellets were placed in individual cages several hours before and maintained in isolated conditions throughout the experimental period. The pellets were implanted s.c. by first pinching the skin on the back of the mouse, cutting a small hole with scissors at the base of the fingers and then inserting the pellet. This innocuous procedure can be done easily in restrained animals after sufficient surgical skill has been acquired. No general anesthesia is necessary. Throughout the entire period food and water were provided *ad libitum.*

The *d*- amphetamine pellets were kindly prepared by Dr. Robert Gibson, University of California School of Pharmacy, San Francisco.

The formulation of the pellet was 148 mg microcrystalline cellulose, 2.0 mg *d*-amphetamine·HCl calculated as base, 0.75 mg fused silicone dioxide, and 0.75 mg calcium stearate. Placebo pellets were of similar composition but lacked *d*-amphetamine.

Twenty-four hr after pellet implantation, animals were administered i.p. various doses of *d*-amphetamine sulfate (calculated as base) dissolved in distilled water. Motor activity and stereotyped behaviors were then assessed for 1 hr as described below. In some experiments, 0.5 hr after the injection of *d*-amphetamine, animals were administered 1 μCi/g body weight of $^3$H-tyrosine (specific activity 11.4 Ci/mmol). Animals were sacrificed 15 and 30 min later by decapitation and the brains removed. The cortex and cerebellum were dissected away and the remainder of the brain, the diencephalon-midbrain-brain stem (DMB) was immediately homogenized in 10 volumes of 0.4 N perchloric acid. The homogenate was then processed for the determination of endogenous and $^3$H-catecholamines and tyrosine as described in Section 3.9. In other experiments, the effects of pellet implantation per se on motor activity and brain amines were determined. For catecholamine determinations, animals were sacrificed every 2 hr after pellet implantation, brains removed, and the whole brain was quickly frozen on dry ice. The pellet was carefully removed from the carcass to determine residual pellet amphetamine levels.

## 6.3 EXPERIMENTAL - BEHAVIORAL.

Locomotor activity and stereotyped behavior were assessed using both the Pharmacia motility meter and the open-field apparatus (see Section 3.12).

### 6.4 RELATIONSHIP OF MOTOR ACTIVITY, BRAIN CATECHOLAMINE LEVELS AND AMPHETAMINE LEVELS AFTER *d*-AMPHETAMINE PELLET IMPLANTATION.[1]

The time course of changes in amine levels and motor activity after pellet implantation is given in Fig. 23. Animals in these and subsequent experiments were implanted only at 9 a.m. in order to minimize the effects of circadian rhythms in catecholamine levels and motor activity. The data in Fig. 23 illustrate that the maximum depletion of brain DA (54 percent) and brain NE (71 percent) occurred 4 and 6 hr after pellet implantation, respectively. The levels of both DA and NE returned to nearly normal values within 12 to 14 hr, but complete recovery was not observed even by 24 hr. Motor activity was increased for the first 2 to 4 hr, gradually declined over the next 14 hr, and finally a marked depression of activity was observed 24 hr after implantation. Brain *d*-amphetamine levels rapidly increased with a level of 84 μg/g being measured 2 hr after implantation. However, by 24 hr after implantation, no *d*-amphetamine was detectable in the brain or in the pellet. Despite the initial high level of *d*-amphetamine, deaths were observed in only 2 to 5 percent of the animals implanted.

### 6.5 EFFECT OF 24 HOUR PELLET IMPLANTATION ON AMPHETAMINE-INDUCED BEHAVIORS.

Since at 24 hr after pellet implantation no *d*-amphetamine was detectable in the brain or the pellet, this time was used to assess the effects of a subsequent dose of *d*-amphetamine on locomotor

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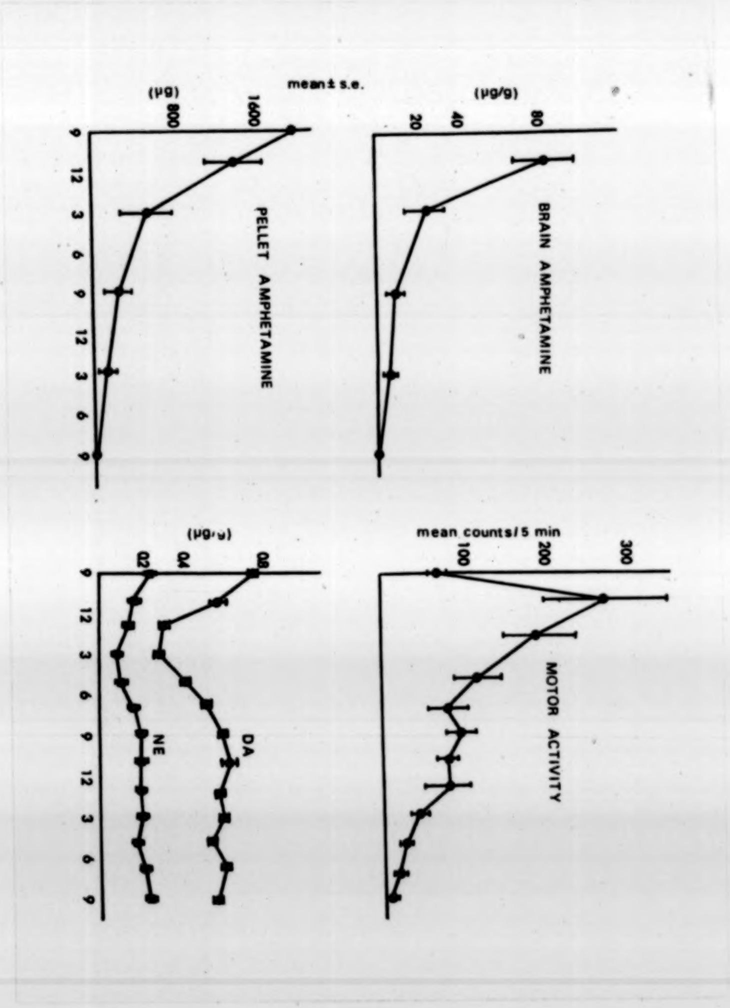
[1] The data described in Fig. 23 and the upper graph of Fig. 26 are from a previous study (Hitzemann, 1970) and were included in this dissertation for the purpose of giving the reader a clearer understanding of the pharmacology of the *d*-amphetamine pellet.

Fig. 23. Relation of brain *d*-amphetamine and catecholamine levels and motor activity as a function of time of pellet implantation. The graphs show the relation of cerebral *d*-amphetamine levels and disappearance of *d*-amphetamine from the pellet to motor activity and brain biogenic amines for 9 A.M. implanted mice. The pellets were implanted s.c. by inserting the pellet under the skin of the back. The formulation of the pellet was 148 mg microcrystalline cellulose, 2.0 mg *d*-amphetamine and HCl calculated as base, 0.75 mg fused silicone dioxide and 0.75 mg calcium stearate. Amine levels are presented in mean ± S.E. in μg/g except for the pellet which is in μg. Motor activity was measured on a Pharmacia MP/40 Fc motility meter for a 5 min trial at the times indicated. Data is expressed as mean counts ± S.E./5 min. N = 10-12 animals/group.


BRAIN AMPHETAMINE
(µg/g)
80
40
20
PELLET AMPHETAMINE
mean ± s.e.
(µg)
1600
800
9
12
3
6
9
12
3
6
9
MOTOR ACTIVITY
mean counts/5 min
300
200
100
DA
NE
(µg/g)
.08
.04
.02
9
12
3
6
9
12
3
6
9

activity. Two methods of measuring motor activity were employed. When the activity meter was used, the activity measured was not only exploratory activities but also some stereotyped short distance locomotive behaviors. The latter behavior has been described in detail by other investigators (Lat, 1965; Randrup and Munkvad, 1970). The second measure of activity employed the open-field apparatus where the animals were scored for both exploratory movements and stereotyped behaviors.

Fig. 24 illustrates that the 24 hr implantation of a single pellet potentiated the effects of challenging doses of 3 and 10 mg/kg but not 30 mg/kg of *d*-amphetamine on activity when the photocell device was used. This potentiation of effect seemed to exhibit an inverse dose-response relationship with 3 mg/kg showing greater potentiation than 10 mg/kg. In some experiments, *d*-amphetamine was administered to 24 hr pellet implanted animals in doses up to 80 mg/kg. While the animals were able to tolerate these high doses fairly well, a second peak of increased motor activity was not observed. Since the *d*-amphetamine disappeared from the pellet fairly rapidly, it was considered possible that only more prolonged *d*-amphetamine levels or repeated pellet implantation would produce tolerance. Fig. 25 demonstrates that when animals were implanted with additional pellets 24 hr after the first and so on, tolerance still did not develop to a challenging dose of 3 mg/kg on activity nor was the potentiating effect reversed.

Tolerance was observed when the open-field test was employed. Fig. 26 illustrates that the 24 hr implantation of a single amphetamine pellet to isolated mice produced a fourfold tolerance to the effects of subsequent doses of *d*-amphetamine on open field activity. The tolerant animals exhibited a bell-shaped dose-response curve as did the

Fig. 24. Effect of pellet implantation on the activity induced by challenging doses of *d*-amphetamine. Mice were implanted with a *d*-amphetamine pellet 24 hr prior to the administration of various doses of *d*-amphetamine i.p. Activity was measured for 1 hr on the motility meter. N = 8 animals/group.

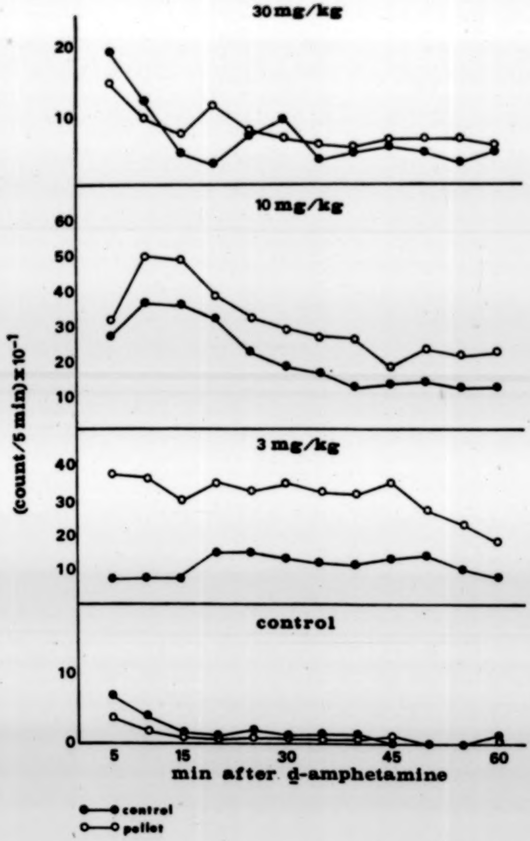
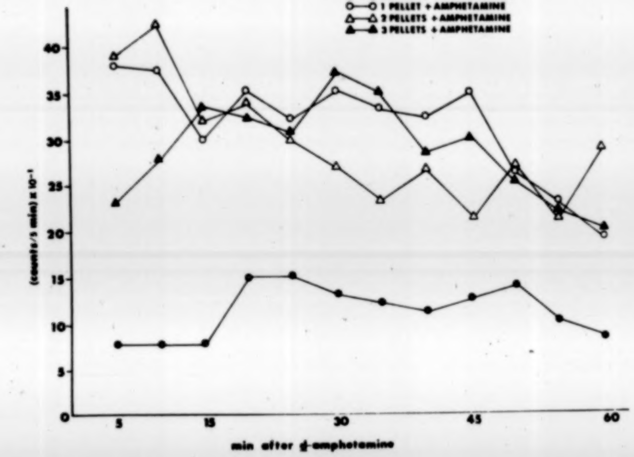
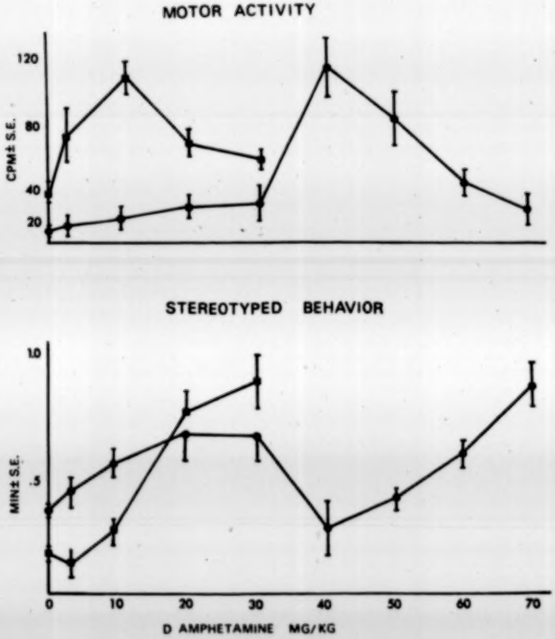

30 mg/kg
20
10
10 mg/kg
60
50
40
30
20
10
3 mg/kg
40
30
20
10
control
10
0
(count/5 min) x 10⁻¹
5
15
30
45
60
min after d-amphetamine
control
pellet

Fig. 25. Effect of multiple pellet implantation on the activity induced by a challenging dose of *d*-amphetamine. Mice were implanted with a single *d*-amphetamine pellet daily for 3 days. Twenty-four hr after each implantation, the animals were administered a challenging dose of *d*-amphetamine (3 mg/kg) i.p. Control animals implanted with multiple placebo pellets showed little difference in activity after implantation and the data from this group was pooled. Activity was measured for 1 hr after amphetamine using the motility meter.


● — ● CONTROL + AMPHETAMINE
○ — ○ 1 PELLET + AMPHETAMINE
△ — △ 2 PELLETS + AMPHETAMINE
▲ — ▲ 3 PELLETS + AMPHETAMINE
(counts/5 min) x 10⁻¹
40
35
30
25
20
15
10
5
0
5
15
30
45
60
min after d-amphetamine

XUM

Fig. 26. Effect of pellet implantation plus challenging doses of *d*-amphetamine on exploratory activity and stereotyped behavior as measured in the open-field. Mice implanted for 24 hr with either placebo or amphetamine pellets were administered various doses of *d*-amphetamine i.p. One hr later exploratory activity and stereotyped behavior were assessed in the open-field (Section 3.12). N = 8-10 animals per group. Δ - amphetamine pellet; □- placebo pellet.



MOTOR ACTIVITY

STEREOTYPED BEHAVIOR

controls with the maximum effective dose for increasing activity being 40 mg/kg. Stereotyped behavior developed more rapidly in the pellet implanted animals with the total time spent in these activities being significantly greater after challenge doses of 3 and 10 mg/kg of *d*-amphetamine. The peak stereotypic behavior response was, however, not significantly different between the pellet and control groups. Interestingly, a decrease in stereotyped behavior was observed at those doses of *d*-amphetamine which caused increased open-field activity in the pellet implanted animals. Although the doses of *d*-amphetamine used in the open-field experiments were fairly large, the mortality rate was less than 5 percent.

### 6.6 EFFECT OF PELLET IMPLANTATION ON THE CONVERSION OF $^3$H-TYROSINE TO $^3$H-CATECHOLAMINES.

In order to examine changes in catecholamine synthesis occurring after pellet implantation, 24 hr implanted animals were administered either saline or *d*-amphetamine (10 mg/kg) i.p. 0.5 hr prior to the i.v. injection of 1 μCi/g body weight of $^3$H-tyrosine (specific activity 11.4 Ci/mmol). Animals were sacrificed 15 and 30 min later and the levels of endogenous- and $^3$H-tyrosine, NE and DA were determined in the diencephalon-midbrain-brain stem (DMB).

The administration of 10 mg/kg of *d*-amphetamine i.p. 24 hr after pellet implantation produced a significant decrease in the levels of DA and NE in the DMB (Fig. 27). In contrast, the levels of DA and NE showed a small but non-significant increase in control animals.

Fig. 28 illustrates the effects of various drug treatments on tyrosine specific activity and the levels of catecholamine radioactivity. In control animals, *d*-amphetamine significantly increased the accumula-

Fig. 27. Effect of pellet implantation plus a challenging dose of *d*-amphetamine on the brain levels of tyrosine, NE and DA. Animals implanted for 24 hr with either *d*-amphetamine or placebo pellets were challenged with 10 mg/kg of *d*-amphetamine i.p. 0.5 hr later the animals were administered 1 μCi/g of $^3$H-tyrosine (specific activity 13.3 Ci/mmol) i.v. The animals were sacrificed 15 and 30 min later and the levels of endogenous plus $^3$H-tyrosine, DA and NE were determined. The data in this figure are from 30 min after tyrosine. * - Significantly different than control - $p < 0.05$. N = 9-10 animals/group.

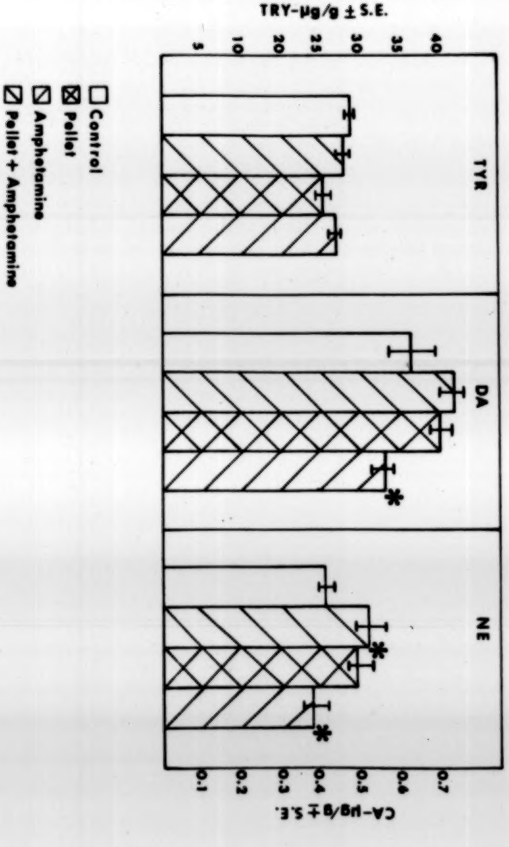
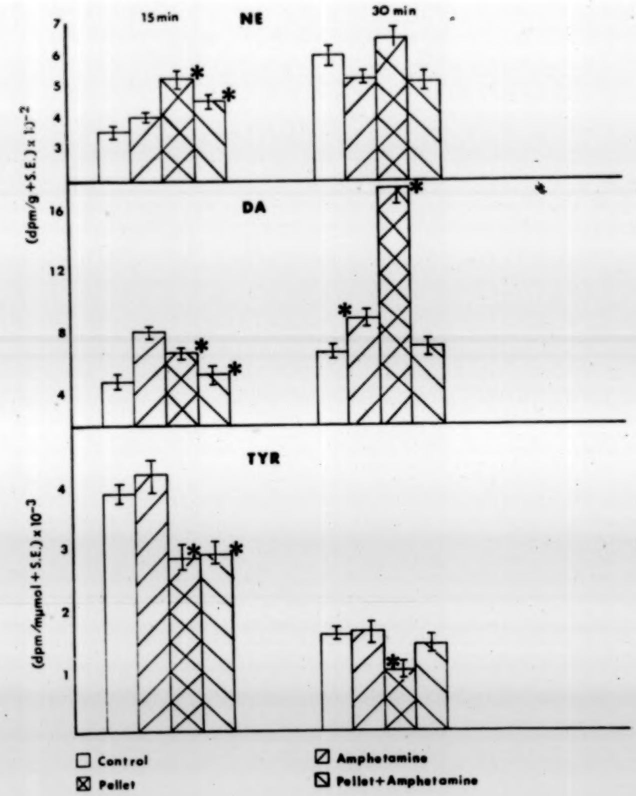

TRY-µg/g ± S.E.
40
35
30
25
20
10
5
TYR
DA
NE
*
*
*
0.7
0.6
0.5
0.4
0.3
0.2
0.1
CA-µg/g ± S.E.
Control
Pellet
Amphetamine
Pellet + Amphetamine

Fig. 28. Effect of pellet implantation plus a challenging dose of *d*-amphetamine on the brain levels of $^3$H-tyrosine, $^3$H-DA and $^3$H-NE after the i.v. injection of $^3$H-tyrosine. Details of the experimental procedure are given in the legend to Fig. 27. Data are presented as mean ± S.E. of tyrosine specific activity and catecholamine radioactivity. N = 9-10 animals per group.


15 min
NE
30 min
7
6
5
4
3
DA
16
12
8
4
(dpm/g + S.E.) x 10^-2
TYR
4
3
2
1
(dpm/mμmol + S.E.) x 10^-3
*
Control
Pellet
Amphetamine
Pellet + Amphetamine

tion of $^{3}$H-DA but had no effect on $^{3}$H-NE levels at 30 min. Twenty-four hr pellet implantation alone increased $^{3}$H-DA levels at 15 min 40 percent ($p < 0.05$) and 130 percent ($p < 0.05$) at 30 min. In this group, $^{3}$H-NE levels were significantly increased at 15 min only. The effects of pellet implantation on $^{3}$H-DA accumulation were reversed by the administration of *d*-amphetamine.

As seen in Fig. 28, tyrosine specific activity was significantly altered by 24 hr pellet implantation. As previously described (Section 2.12), the conversion index is a useful calculation for incorporating this type of change into the evaluation of drug effects of $^{3}$H-catecholamine accumulation. The CI values for the various drug treated groups are given in Table 9. The CI was increased 330 percent for $^{3}$H-DA and 61 percent for $^{3}$H-NE in the pellet group at 30 min after $^{3}$H-tyrosine administration. *d*-Amphetamine antagonizes the increased CI values observed in the 24 hr pellet animals but increases the CI for DA in control animals.

## 6.7 EFFECT OF α-MT ON *d*-AMPHETAMINE INDUCED STEREOTYPED BEHAVIORS IN NORMAL AND PELLET IMPLANTED ANIMALS.

In mice administered α-methyl tyrosine (α-MT), 200 mg/kg i.p. 22 hr after pellet implantation, the amphetamine-induced stereotyped behaviors were significantly decreased (Table 10).

## 6.8 DISCUSSION.

The mechanism(s) by which pellet implantation causes tolerance development to the amphetamine-induced increase in exploratory locomotor activities are presently unclear. On the basis of the observations that neither α-MT and/or reserpine pretreatments alter this

## TABLE 9

### Effect of Various Drug Treatments on the Conversion of $^{3}$H-Tyrosine to $^{3}$H-Catecholamines [a]

| | | Conversion Index | |
|---|---|---|---|
| | | DA | NE |
| Group | Time | mμmol/g/min | |
| Control | 15 | 0.08 | 0.06 |
| | 30 | 0.14 | 0.13 |
| *d*-Amphetamine-10 mg/kg | 15 | 0.12 | 0.06 |
| | 30 | 0.19 | 0.11 |
| Pellet | 15 | 0.16 | 0.13 |
| | 30 | 0.61 | 0.21 |
| Pellet + *d*-Amphetamine 10 mg/kg | 15 | 0.15 | 0.11 |
| | 30 | 0.16 | 0.12 |

[a] The schedule of drug and $^{3}$H-tyrosine administration is given in the legend to Fig. 27. The details of the calculation of the conversion index are given in Section 2.12.

## TABLE 10

### Effect of α-MT on the Potentiation of Amphetamine Stereotypes in Pellet Implanted Animals[a]

| | Stereotyped Activity | |
|---|---|---|
| | Min ± S.E. | |
| Group | Vehicle | α-MT |
| Placebo | 0.23 ± 0.05 | 0.14 ± 0.01 |
| Placebo + *d*-Amphetamine | 0.38 ± 0.06 | 0.19 ± 0.02 |
| Pellet | 0.48 ± 0.06 | 0.21 ± 0.05 |
| Pellet + *d*- Amphetamine | 0.85 ± 0.07 | 0.29 ± 0.06 |

[a] Animals were implanted with placebo or amphetamine pellets. Twenty-two hr later the animals were administered 200 mg/kg α-MT or the α-MT vehicle. Two hr later the animals were challenged with either saline or 10 mg/kg i.p. *d*-amphetamine. One hr later the animals were given a single 5 min trial in the open-field and the minutes the animals engaged in stereotyped activity was assessed. N = 6 to 8 animals per group.

phenomenon, it was previously concluded that an indirect mechanism involving the catecholamines was unlikely (Hitzemann, 1970). However, certain lines of evidence in the present study suggest that the mechanism by which 24 hr pellet implantation potentiates the effectiveness of *d*-amphetamine to induce stereotyped behaviors is related to an indirect effect on brain DA. This evidence may be summarized: (a) α-MT pretreatment reduced the effectiveness of *d*-amphetamine to produce stereotyped behaviors in 24 hr pellet implanted animals; (b) pellet implantation markedly increased the synthesis of $^3$H-DA formed from $^3$H-tyrosine.

The quantitative assessment of the effects of pellet implantation on the development of amphetamine stereotypes was obtained by observing the animals in the open field. Some qualitative verification of the potentiation or negative tolerance caused by implantation was obtained with a photocell device. The close proximity of the photocells ensures that locomotor and non-locomotor stereotypes as well as exploratory activity will be counted. While exploratory activities may register quantitatively, the stereotypes apparently register only semi-quantitatively. For example, in the placebo treated mice both the open field and the photocell device gave a bell-shaped dose-response curve for amphetamine. The increase in stereotypes (primarily non-locomotor) seen at 30 mg/kg was counted on the photocell device as a decrease in movement when compared to 10 mg/kg. However, in the 24 hr pellet implanted animals the photocell device more quantitatively measured the increase in stereotyped locomotor movements (see Lat, 1965, and Randrup and Munkvad, 1970) at 3 mg/kg. These animals did not exhibit any appreciable exploratory activities. Overall, it appears that results from the photocell device must be interpreted with great caution. By removing most of the photocells except for one medial row (as in Chapter 4), the data obtained seem to more closely approximate the results obtained with the open-field (preliminary observation). However, it is this author's conclusion that any behavioral results obtained with motility meters should be verified by direct observation of the animal's behavior.

Fuxe and Ungerstedt (1970) have presented evidence that the amphetamine-induced increase in exploratory activity is related to an increase in noradrenergic receptor activity in the hypothalamus and limbic system while the stereotyped behaviors can be attributed to an activation of brain dopaminergic neurons in the neostriatum (Randrup and Munkvad, 1970). From these data, the authors postulated that the ratio of DA to NE receptor activity will determine the nature of the animal's response to *d*-amphetamine. A similar theory was presented in Section 4.9. From these models, one could conclude that in pellet implanted animals receiving challenging low doses of *d*-amphetamine the ratio of DA to NE receptor activity increases.

To test this hypothesis, the effect of pellet implantation plus challenges of *d*-amphetamine on the cerebral conversion of $^3$H-tyrosine to $^3$H-catecholamines and on changes in endogenous catecholamine levels was examined. Costa and Groppetti (1970) and Persson (1969) have shown that acute *d*-amphetamine treatment increases the conversion of a pulse label $^3$H-tyrosine to $^3$H-DA but not $^3$H-NE in the rat brain. The present study confirms that this effect also occurs in the mouse brain. This increased conversion to $^3$H-DA is thought to represent an increase in brain DA turnover (Costa and Groppetti, 1970).

Pellet implantation seemed to cause an accumulation of *d*-amphetamine's effects on $^3$H-DA formation. In 24 hr implanted animals, the CI for DA was increased 330 percent while the CI for NE was increased only 61 percent. If an increase in conversion truly is representative of an increase in turnover and, consequently, an increase in transmitter release, these data would suggest that the ratio of DA/NE receptor activity is increased after pellet implantation. These data may help to explain the increase in stereotyped behavior in pellet implanted animals receiving no additional *d*-amphetamine (see Fig. 26).

In 24 hr pellet implanted animals administered 10 mg/kg of *d*-amphetamine, no change from control was observed in the conversion of $^3$H-tyrosine to $^3$H-catecholamines. The mechanism by which a single injection of *d*-amphetamine can reverse the increased conversion observed in the pellet controls may be related to the rapid decrease in endogenous catecholamine levels (Table 10). This attenuated mobilization of catecholamines from intraneuronal storage vesicles could reverse the increase in $^3$H-catecholamine synthesis by increasing the levels of catecholamines in the nerve-ending cytosol which compete with the reduced pteridine cofactor for the oxidized tyrosine hydroxylase (Ikeda *et al.*, 1966; Kopin *et al.*, 1968; Weiner *et al.*, 1972).

However, if DA synthesis is normal in the pellet implanted animals challenged with *d*-amphetamine, it is difficult to explain the potentiation of the stereotyped behaviors unless we assume that it is related to the rapid mobilization of DA stores. This conclusion, though, is contrary to the observation that α-MT blocks the stereotyped behaviors. The solution to this problem is presently not evident. One possibility is that the blockade caused by α-MT is related to the sedative effects of the drug even though in normal mice and rats this is not the case (see Chapter 2).

Overall, the data indicate that acute *d*-amphetamine intoxication enhances the ability of subsequent doses of *d*-amphetamine to elicit DA related behaviors. Interestingly, the behavioral effect obtained is similar to that caused by chronic PONE administration (Chapter 5).

## 6.9 GENERAL COMMENTS.

One problem with the study described in this chapter is the possibility that the control may be inappropriate. Both placebo and amphetamine pellet implanted animals were placed in isolated conditions several hours before and throughout the entire experimental procedures. This was done to reduce the aggregation toxicity that occurs when large doses of amphetamine are administered. In the amphetamine pellet implanted animals it seems reasonable that the drug effect predominates over isolation-induced aggression. However, in the placebo group the isolation-induced aggression is a factor that can influence brain catecholamine levels (Weil-Malherbe, 1972). Thus, the control is not really a control but rather a "treated" group. Perhaps the more appropriate control would be group housed animals. This problem of what is the appropriate control for isolated amphetamine treated animals is a subject that is rarely considered but one that must at least be kept in mind when studies such as the kind described in this chapter are performed.

The data presented in this chapter indicate that acute *d*-amphetamine treatment enhances the accumulation of $^3$H-DA and has no effect on $^3$H-NE accumulation when $^3$H-tyrosine is administered i.p. These data are similar to the results obtained by Costa and Groppetti (1970) in the rat. However, the data are in marked contrast to the results obtained in Chapter 5 where *d*-amphetamine was found to inhibit $^3$H-NE accumulation when $^3$H-tyrosine was administered intracisternally. Thus, the route of precursor administration dramatically influences the effect of *d*-amphetamine on the conversion of $^3$H-tyrosine to $^3$H-catecholamines. The reasons for these differences are presently not clear. Perhaps when $^3$H-tyrosine is administered i.p., *d*-amphetamine influences the uptake of $^3$H-tyrosine to the sites of catecholamine synthesis. It would be interesting to know if *d*-amphetamine increases the specific activity of $^3$H-tyrosine in the "heavy" (see Chapter 3) nerve ending particles. The data in this chapter indicate that *d*-amphetamine enhances $^3$H-tyrosine specific activity in the DMB. The possibility must also be considered that the direct injection of $^3$H-tyrosine produces an abnormal distribution of label. Obviously, the solution to this dilemma would be of great interest to all investigators studying the effect of drugs on brain catecholamine synthesis.

In Chapter 4 the concept was developed that the ratio of DA to NE receptor activity determines the nature of the behavioral response. The behavioral data in Chapter 5 indicate that PONE increases the ratio such that challenging doses of *d*-amphetamine cause primarily stereotyped activity. The behavioral and chemical data presented in this chapter suggest that amphetamine intoxication in the mouse, a species which does not metabolize *d*-amphetamine to PONE, increases the DA to NE receptor activity ratio. In the next chapter the effect of chronic amphetamine administration in the rat on behavior and the brain catecholamines will be examined; the data accumulated to this point suggest a hypothesis which dould predict that chromic amphetamine administration in the rat will increase the receptor activity ratio.

# CHAPTER 7

# CHRONIC AMPHETAMINE INTOXICATION IN THE RAT

## 7.1 INTRODUCTION.

The study of amphetamine intoxication in the rat is somewhat complicated from an interpretive viewpoint by the fact that two pharmacologically active amphetamine metabolites, namely p-OH-amphetamine and PONE (Taylor and Sulser, 1973) accumulate in the brain. Since these metabolites are released with subsequent amphetamine injections, it is likely they will have important effects on behavior and, consequently, on the relevant neurochemical and neurophysiological processes involved (see Chapter 5). In the present chapter the effects of chronic amphetamine injections in the rat on exploratory activity, stereotyped behavior and catecholamines are described.

The design of the study was such that the effects of the metabolites will be diminished. The animals were administered amphetamine for two weeks on a staircase schedule and the influence of these injections on the effects of challenging doses of *d*-amphetamine was determined for one week after the last chronic injection. With the methods employed, no amphetamine metabolites were detected 48 hr after the last injection and only trace amounts were found at 24 hr. Thus, any behavioral and neurochemical changes observed during the 7 days after discontinuing the chronic injections should primarily be the result of the chronic amphetamine injections per se or perhaps the result of "permanent" alterations induced by the combination of amphetamine and amphetamine metabolites.

Historically, there are relatively few studies on the effects of chronic amphetamine injections in the rat. Repeated injections of amphetamine have been found to produce tolerance to the drug's effects on some complex behaviors (Schuster and Zimmerman, 1961; Fischman and Schuster, 1974; Schuster *et al.*, 1966), on hyperthermia (Brodie *et al.*, 1970) and on anorexia (Harrison *et al.*, 1952). Reports indicating that tolerance develops to amphetamine-induced psychomotor stimulation have been positive (Yagi, 1963) and negative (Brodie *et al.*, 1969; Schuster and Zimmerman, 1961). Gunne and Lewander (1968) and Lewander (1968) have shown that short-term chronic amphetamine treatment in rats caused a gradual depletion of catecholamines in the heart and brain and an increased urinary excretion of catecholamines. With long-term amphetamine injections, these authors found that urinary catecholamine levels began to return to normal, suggesting that some tolerance had developed to the amphetamine effects. Javoy *et al.* (1968) have observed that chronic (4 days) treatment with a moderate dose of *d*-amphetamine had no effect on NE turnover in two regions of the rat brain.

## 7.2 EXPERIMENTAL - CHEMICAL.

Male Sprague-Dawley rats (175-200 g) obtained from Simonsen Laboratories, Gilroy, Cal., were used in all experiments. Animals were administered *d*-amphetamine i.p. chronically using a staircase schedule. Injections were begun at 1 or 3 mg/kg and the dose was doubled every 5 days. The injections were terminated after 15 days. Beginning 24 hr after the last injection and continuing for a week thereafter, animals were sacrificed and three aspects of catecholamine metabolism were analyzed. *In vitro* catecholamine synthesis and release were examined in tissue slices prepared from the cortex, brain stem and caudate nucleus using the methodology of Harris and Roth (1971) (see Section 3.13). Catecholamine uptake was investigated in the above brain regions using both the tissue slice and crude homogenate techniques (see Section 3.9). In some animals the last amphetamine injection contained 10 μCi of $^3$H-amphetamine/mg amphetamine. Animals were sacrificed 2, 24 and 48 hr thereafter and brain tissue was analyzed for $^3$H-amphetamine, $^3$H-p-OH-amphetamine and $^3$H-PONE (see Section 3.14).

## 7.3 EXPERIMENTAL - BEHAVIORAL.

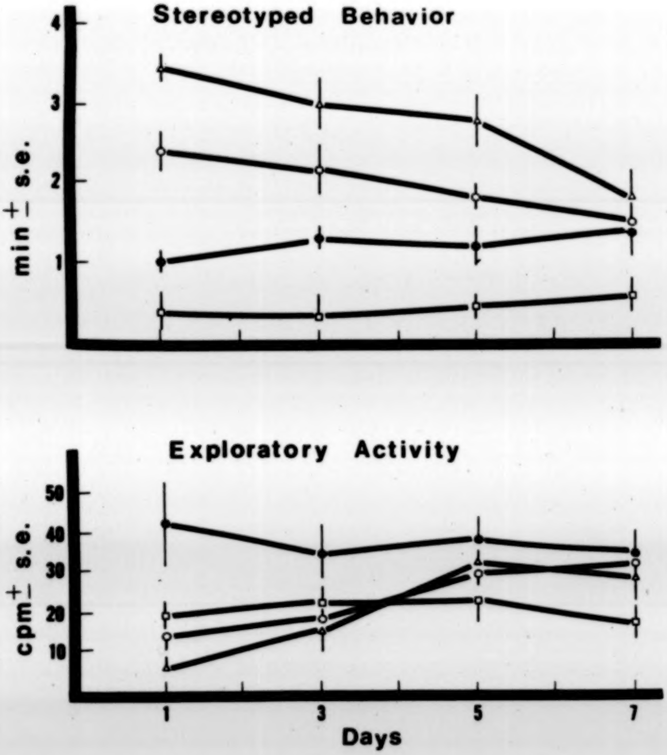
Using the drug injection and withdrawal schedule previously described, the effects of chronic amphetamine injections to modify the actions of challenging doses of *d*-amphetamine on exploratory activity and stereotyped behavior were analyzed in the open-field (see Section 3.12).

## 7.4 EFFECT OF CHRONIC AMPHETAMINE TREATMENT ON EXPLORATORY ACTIVITY AND STEREOTYPED BEHAVIORS.

The chronic administration of *d*-amphetamine on both the high and low dose schedule decreased or eliminated the ability of a challenging drug dose to increase exploratory activity while at the same time enhanced amphetamine's effectiveness to induce stereotyped behaviors (Fig. 29). These effects of the chronic injections were most pronounced on days 1 and 3. By day 5, the ability of *d*-amphetamine to increase exploratory activity had partially returned and the potentiation of the stereotyped behaviors was diminished. As would be expected, the return to the normal drug response was most obvious for those animals chronically administered amphetamine on the low dose schedule. On day 7 both groups of chronically treated animals showed nearly normal drug responses.

Fig. 29. Effect of chronic amphetamine treatment on acute *d*-amphetamine-induced changes in stereotyped behavior and exploratory activity. Animals were administered *d*-amphetamine chronically on a high and low dose staircase schedule (see Methods). One, 2, 5 and 7 days after the last injection the animals were challenged with 5 mg/kg of *d*-amphetamine i.p. and exploratory activity and stereotyped behavior was assessed in the open-field (Section 3.12). □ - no drug; ● - control + 5 mg/kg of *d*-amphetamine; 0 - low dose chronic + 5 mg/kg of *d*-amphetamine, Δ - high dose chronic + 5 mg/kg of *d*-amphetamine. No drug data for the two groups of chronically treated animals was not significantly different than the no drug control (data not shown).

XUM


Stereotyped Behavior
min ± s.e.
4
3
2
1
Exploratory Activity
cpm ± s.e.
50
40
30
20
10
1
3
5
7
Days

## 7.5 DISAPPEARANCE OF *d*-AMPHETAMINE, p-OH-AMPHETAMINE AND PONE FROM THE CHRONICALLY TREATED ANIMALS.

The data in Table 11 illustrate that $^3$H-p-OH-amphetamine and $^3$H-PONE had almost completely disappeared from the brain 48 hr after injection of $^3$H-amphetamine. $^3$H-Amphetamine levels at 24 hr were only slightly above background.

## 7.6 EFFECT OF CHRONIC AMPHETAMINE TREATMENT ON *In vitro* CATECHOLAMINE SYNTHESIS.

Based on the information obtained from the experiments described in the previous chapters, it appeared that the *in vitro* technique (see Harris and Roth, 1971) of measuring catecholamine synthesis in tissue slices gave the readily interpretable data and perhaps the most accurate as well. For this reason, the *in vitro* technique was solely employed in this study to assess the effects of chronic amphetamine treatment on catecholamine synthesis.

The data in Fig. 30 show that 24 hr after discontinuing the chronic amphetamine treatment, the levels of endogenous NE were significantly decreased in the cortex and brain stem in both the high and low dose schedule; DA levels in the neostriatum were depressed 28 percent in the high dose schedule. By day 3 endogenous catecholamine levels returned to normal and were still normal at 7 days.

At 24 hr, chronic amphetamine treatment had distinct effects on catecholamine synthesis and disposition depending on the dose schedule employed (Fig. 31-32). On the low dose schedule, chronic amphetamine enhanced the release of DA in the neostriatum and NE in both the cortex and brain stem without markedly affecting the net (tissue plus medium) synthesis. On the high dose schedule, DA synthesis was decreased 46

TABLE 11

Disappearance of $^3$H-Amphetamine and $^3$H-Amphetamine Metabolites from the Rat Brain [a]

| Group | | Control | Chronic Amphetamine |
|---|---|---|---|
| | | cpm/g | |
| $^3$H-Amphetamine | 2 hr | 5639 | 6500 |
| | 24 hr | 125 | 210 |
| | 48 hr | N.D. | N.D. |
| $^3$H-p-OH-Amphetamine | 2 hr | 2100 | 2630 |
| | 24 hr | 432 | 569 |
| | 48 hr | 51 | 37 |
| $^3$H-PONE | 2 hr | 429 | 311 |
| | 24 hr | 365 | 386 |
| | 48 hr | 17 | 31 |

[a] Control or chronic amphetamine (high) treated rats were administered 10 mg/kg of $^3$H-*d*-amphetamine (specific activity 10 μCi/mg) on day 15 (see methods for details of injection schedule). The animals were sacrificed at various times thereafter and $^3$H-amphetamine and $^3$H-amphetamine metabolite levels were determined in the whole rat brain. Data are the mean value of two experiments.

Fig. 30. Effect of chronic amphetamine treatment on endogenous DA and NE levels in some discrete regions of the rat brain. Animals were administered *d*-amphetamine chronically on the high and low dose staircase injection schedule described in methods. One, 3 and 7 days later the animals were sacrificed and the levels of endogenous DA in the caudate nucleus and NE in the brain stem and cortex were determined. N = 4 to 6 determinations per group. ● - Control; O - Low dose schedule; Δ - High dose schedule.

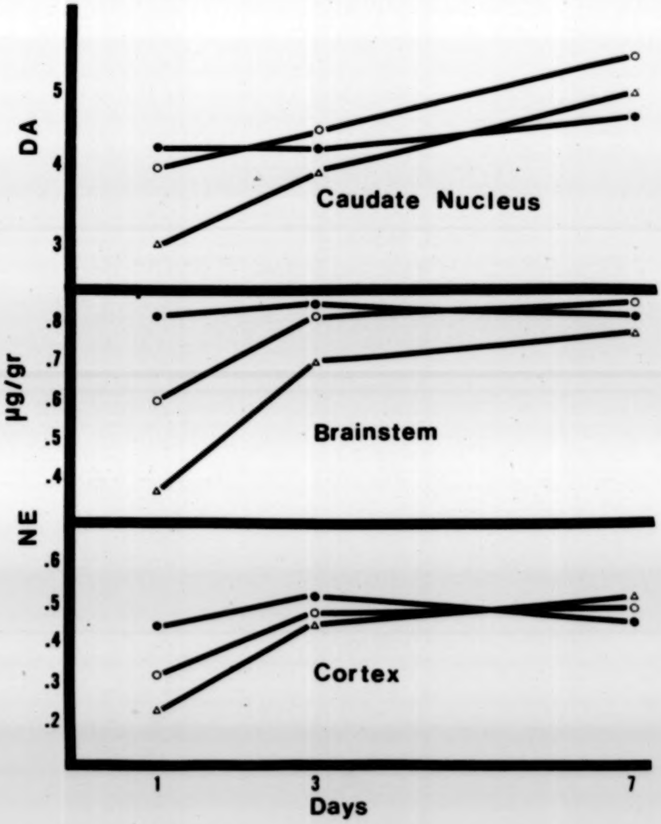

DA
5
4
3
Caudate Nucleus
μg/gr
.8
.7
.6
.5
.4
Brainstem
NE
.6
.5
.4
.3
.2
Cortex
1
3
7
Days

Fig. 31. Effect of chronic amphetamine administration on *in vitro* NE synthesis in the brain stem and cortex. Rats were administered *d*-amphetamine chronically for 15 days on a low and high dose schedule as described in methods. On days 1, 4 and 7 after the last injection, the animals were sacrificed and tissue slices (0.26 mm) were prepared from the cortex and brain stem. Slices were incubated in the presence of $2 \times 10^{-5}$ M $^{3}H$-tyrosine as described by Harris and Roth (1971). The rates of NE appearance in the tissue (T) and medium (M) were determined and the T/M ratio calculated. ● - Control; 0 - Low dose schedule; Δ - High dose schedule.

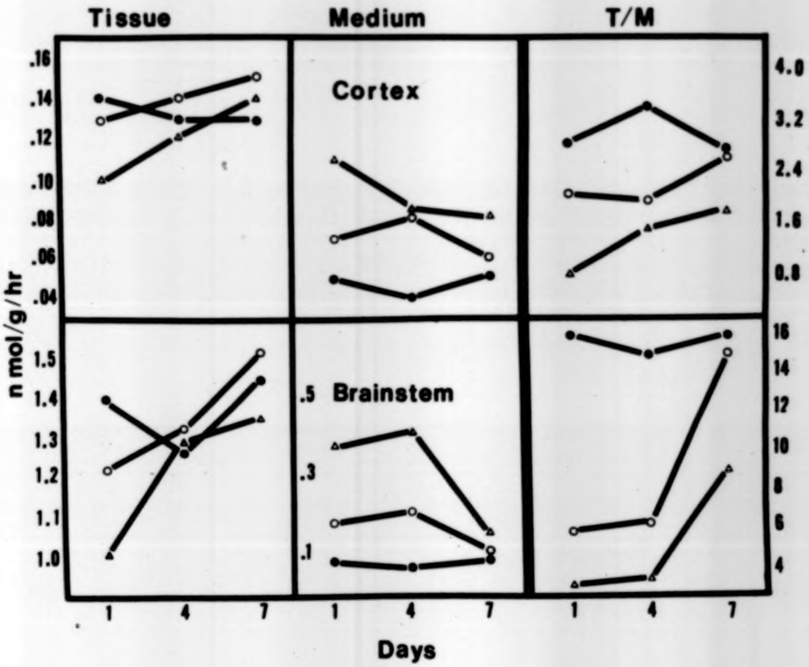
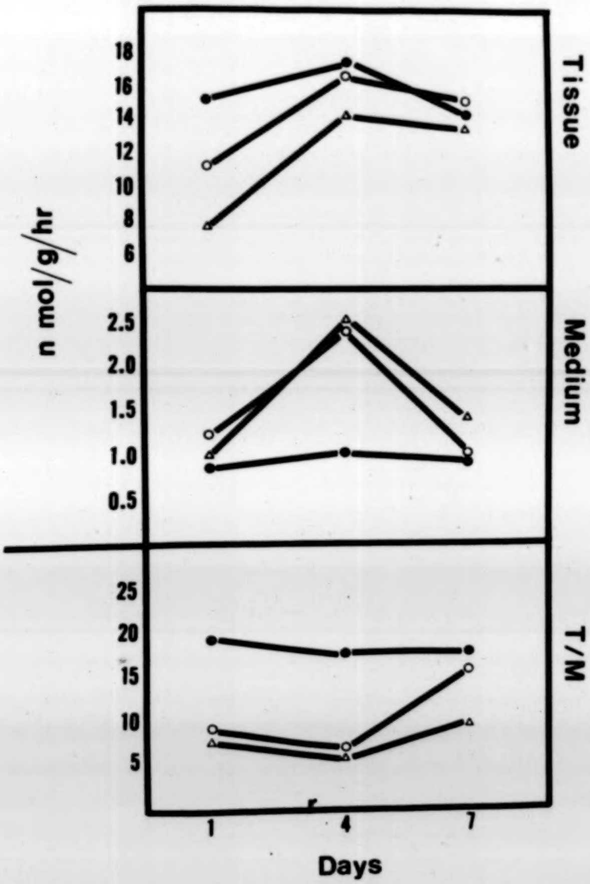

Tissue
Medium
T/M
Cortex
Brainstem
n mol/g/hr
.16
.14
.12
.10
.08
.06
.04
1.5
1.4
1.3
1.2
1.1
1.0
.5
.3
.1
4.0
3.2
2.4
1.6
0.8
16
14
12
10
8
6
4
1
4
7
Days

Fig. 32. Effect of chronic amphetamine administration on *in vitro* DA synthesis in the caudate nucleus. Details of experimental procedures given in legend to Fig. 31.

Caudate Nucleus



percent but the T/M ratio was similar to that observed in the low dose schedule. Somewhat differently, NE synthesis was both depressed and the T/M ratio was further decreased in the cortex and brain stem.

By day 4 catecholamine synthesis had returned to near normal in all brain regions studied. However, the disposition of the catecholamines was still significantly changed in both dose schedules. The T/M ratios were decreased 35 to 50 percent for all groups. On day 7 the T/M ratio for the low dose schedule returned to normal. On the high dose schedule the T/M ratio was depressed 46 percent in the neostriatum, 31 percent in the brain stem and 44 percent in the cortex. These data indicate that residual amphetamine effects on catecholamine disposition persist for at least one week after discontinuance of the drug.

## 7.7 EFFECT OF CHRONIC AMPHETAMINE TREATMENT ON CATECHOLAMINE UPTAKE.

The data reported in the previous section suggest that either chronic amphetamine has modified the spontaneous release of newly synthesized catecholamines and/or that the chronic treatment has caused a "permanent" decrease in catecholamine uptake. To examine the latter possibility, uptake was measured in chronically treated animals using both a tissue slice and a crude homogenate preparation. It was found that chronic amphetamine (high dose schedule) had no effect over the 7 day experimental period on DA uptake in the neostriatum and NE uptake in the cortex and brain stem. Typical data taken from 3 days after withdrawal of amphetamine is illustrated in Fig. 33. Strangely though, when NE uptake was measured in the cortex using the tissue slice preparation, it was observed that uptake was actually enhanced by chronic amphetamine treatment (Fig. 34). This enhancement was maximal on day 1 and disappeared by day 7.

Fig. 33. Graphic analysis of the reciprocals of $^3$H-NE or $^3$H-DA concentration and their accumulation into a particulate fraction isolated from discrete regions of rats treated chronically with amphetamine. Rats were administered *d*-amphetamine chronically as described in the Methods. On day 3 after the last injection, animals were sacrificed and a crude mitochondrial fraction was prepared from the brain stem, cortex and caudate nucleus. The uptake of NE into the cortex and brain stem and DA into the caudate nucleus was determined as described by Snyder and Coyle (1969). Velocity (V) is expressed as (mμmol of $^3$H/mg protein/t min) x $10^{-2}$. Lineweaver-Burke plots were fitted by means of Wilkinson (1961) analysis and represent the mean of at least 4 determinations performed in duplicate. The control data from the various days differed by less than 5 percent and was consequently combined.

0 - 0 = Control; ● - ● = Day 3.

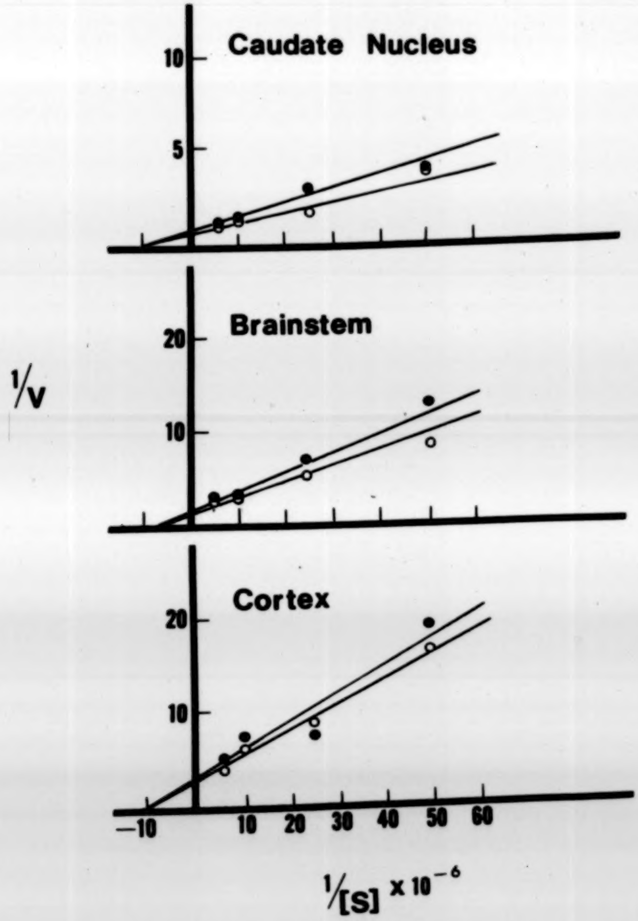
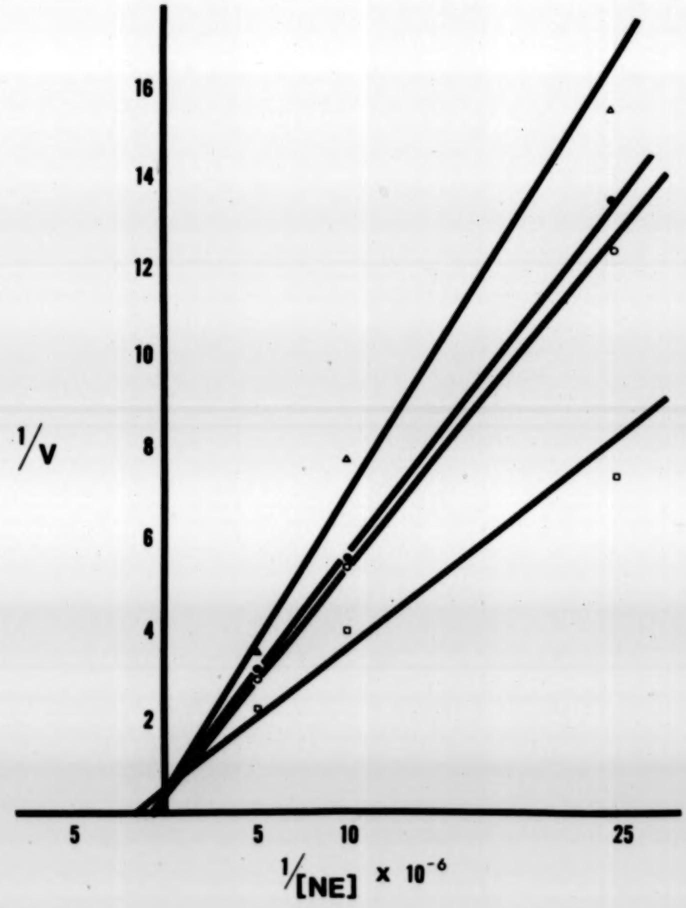

Caudate Nucleus
10
5
Brainstem
20
10
Cortex
20
10
1/v
−10
10
20
30
40
50
60
1/[S] x 10^−6

Fig. 34. Graphic analysis of the reciprocals of $^3$H-NE concentration and accumulation into cortical tissue slices prepared from rats treated chronically with *d*-amphetamine. The details of the experimental procedures are essentially those described in the legend to Fig. 33 except that cortical tissue slices (0.26 mm) were used. Velocity (V) is expressed as (mμmol of $^3$H/g wet tissue/5 min) x $10^{-2}$. The control data from the various days differed by less than 5 percent and was consequently combined. ● - ● = Control; 0 - 0 = Day 1; □-□ = Day 4; Δ Δ = Day 7.


16
14
12
10
8
6
4
2
1/V
5
5
10
25
1/[NE] x 10^-6

## 7.8 DISCUSSION.

The data in this chapter demonstrate that chronic amphetamine intoxication had different effects on catecholamine synthesis depending on the brain region investigated and the dose schedule used. On the low dose schedule at 24 hr after amphetamine withdrawal the release of newly synthesized catecholamines was increased in all brain regions. However, only in the caudate nucleus was catecholamine (DA) synthesis decreased. These data are similar to the results of Beeson *et al.* (1973) who have reported that chronic amphetamine injections in rats decreases catecholamine synthesis in striatal but not cortical brain slices. On the high dose schedule catecholamine synthesis was markedly inhibited in all brain regions at 24 hr. The mechanism(s) by which chronic amphetamine injections influence catecholamine synthesis and release are presently not clear. The observation that endogenous catecholamine levels are depressed at the same times catecholamine synthesis is decreased may be more than coincidence. From the data presented in Chapter 5, it was concluded that acute amphetamine treatment decreases NE synthesis by increasing the intraneuronal level of unbound catecholamine and these increased levels block synthesis via product feedback inhibition. Similarly, if amphetamine intoxication decreases the ability of the granules to store catecholamines, a point supported by the data in this and the previous chapter, the intrasynaptic levels of unbound catecholamines would increase and catecholamine synthesis would decrease.

However, it would seem that the most significant effect observed in the experiments described in this chapter is not the transient decrease in catecholamine synthesis but rather the persistent increase in catecholamine release. The release of the newly synthesized DA and NE is significantly enhanced for at least one week. The increase in release was not an indirect effect resulting from decreased reuptake. In fact, if anything, the data would suggest that chronic amphetamine intoxication enhances reuptake. The increases in release of the newly synthesized catecholamines are not significantly different among the three brain regions investigated. Thus, based on these data, one could not predict that the ratio of DA to NE receptor activity is increased. Based on the behavioral effects of challenging doses of *d*-amphetamine to produce primarily stereotyped behaviors, though, it appears the ratio is increased. Perhaps a percent comparison of the increase in release is not entirely valid. On an amount basis, there is clearly more newly synthesized DA released than newly synthesized NE in either the cortex or brain stem. The dilemma of knowing whether to use amount or percentage awaits at least a clearer understanding of the density of the dopaminergic or noradrenergic nerve endings in the various brain regions.

## 7.9 GENERAL COMMENTS.

This is the last chapter dealing with the effects of amphetamine intoxication on brain catecholamines and behavior. Using three different experimental models, it was found that intoxication causes low challenging doses of amphetamine to produce enhanced stereotyped behaviors rather than increased exploratory activities. Chemically, this shift is accompanied by an increase in the DA to NE receptor activity ratio. In species such as the rat which p-hydroxylate amphetamine, the metabolite PONE could increase the ratio by competing with NE at the receptor site (Chapter 5). PONE does not block NE synthesis as Kopin *et al.* (1968) have demonstrated for other false transmitters in the rat heart. However, in the rat PONE alone cannot account for the shift in behavior since the metabolite disappears from the brain within 48 hr after the last amphetamine injection but the behavioral shift persists for at least one week. Temporally accompanying this persistent shift in behavior, the release of newly synthesized NE and DA is increased with the increased amount of newly synthesized DA released in the caudate being much greater than the amount of newly synthesized NE released in the cortex and brain. In hindsight, one experiment that should have been performed and wasn't would be to test the sensitivity of the DA receptor with the specific receptor stimulant apomorphine. Recently Dr. Tseng in Dr. Loh's laboratory has examined the effect of apomorphine in rats administered amphetamine chronically on a schedule very similar to the high dose schedule described in this chapter. His preliminary results suggest that chronic amphetamine treatments potentiate the ability of apomorphine to induce stereotyped behaviors.

Clinically, one of the most pronounced symptoms of chronic amphetamine abuse is paranoid schizophrenia. Several workers have suggested that the etiology of psychosis is related to a disturbance of DA neurons (Heath *et al.*, 1965; Domino *et al.*, 1968; Janssen, 1968; Janssen *et al.*, 1965; Randrup and Munkvad, 1970). The results described in Chapters 5 to 7 indicate that chronic amphetamine intoxication modifies the function of brain DA neurons. Thus, the possibility must be considered that the abnormal clinic behaviors observed during chronic amphetamine abuse are causally related to a dysfunction of DA neurons.

## CHAPTER 8

## STRUCTURAL-FUNCTIONAL ASPECTS OF NE REUPTAKE AND SPONTANEOUS RELEASE

### 8.1 INTRODUCTION.

In the previous chapter it was established that chronic *d*-amphetamine intoxication produces at least one "permanent" alteration in catecholamine function; namely, increased spontaneous release of newly synthesized DA and NE. In addition to the alteration chronic amphetamine administration produces in the ability of the drug to induce increased exploratory activities and stereotyped behaviors, it is known that the chronic drug treatment causes tolerance to develop to certain drug-induced behaviors, e.g., anorexia, and can cause some signs of physical dependence (Oswald, 1970). It has been observed that amphetamine as well as a variety of other drugs suppress paradoxical or REM sleep, tolerance develops to this effect and upon withdrawal of the drug REM sleep becomes exaggerated. Readministration of the drug blocks this sign of dependency. The exact way the chronic drug induced changes in catecholamine release fit into the aforementioned adaptive and tolerance mechanisms is presently not clear. Some speculation on this subject will be offered in Chapter 12. The question to which this and the succeeding three chapters are directed to answering is what change does *d*-amphetamine produce in the noradrenergic neuron that could possibly account for the persistent alteration in spontaneous release. Investigating the mechanism(s) underlying the change in release will undoubtedly provide important information in our understanding of how the brain adapts to the central effects of amphetamine.

It is assumed that change(s) of a "permanent" nature are related to modifications of membrane components which turnover slowly in the brain, e.g., proteins, phospholipids, glycoproteins, etc. However, before investigating the effects or lack of effects of *d*-amphetamine on these substances, it seems necessary to first establish in at least a superficial way what role these compounds have in NE release. In addition, it would seem prudent to examine the role of these membrane components in the antithetical function of release, reuptake. Since it has been previously established that chronic amphetamine intoxication probably has only minimal effect on reuptake, it would be advantageous to examine some component or components which are primarily involved in release. As often is the case in pharmacological research and as will be seen in this section, that data which is least pertinent to the main argument, in this case the role of membrane components in reuptake, proves to be the most interesting. Thus, for purposes not entirely consistent with our working hypothesis but consistent with the more general theme of the role of membrane components in neurotransmitter function, the structural-functional aspects of reuptake have been examined in some detail.

The approach taken to determine the structural-functional relationship involved in the release and reuptake of NE was to examine the effect of some proteolytic and lipolytic enzymes on these processes. Phospholipases and proteolytic enzymes have been used to study the relationships between membrane structure and function in amino acid, carbohydrate and small ion transport (Kirschmann *et al.*, 1971; Wallach, 1969; Tobias *et al.*, 1962; Blecher, 1965, 1967). The selective action of the various enzymes on membrane proteins and phospholipids enables the investigator to determine active and non-active components or sites in the membrane required for the transport process. These types of studies have, however, only rarely been performed in conjunction with transport in brain tissue. In one study, Kirschmann *et al.* (1971) have found that treatment of mouse brain slices with phospholipases A and C, but not trypsin, inhibited histidine uptake.

The role of phospholipids in the release of catecholamines via exocytosis has been discussed previously (Chapter 2). Briefly, it is suggested that the membranes of the synaptic vesicles will fuse with the cytoplasmic membrane of the nerve ending and this process is initiated by the action of the detergent lysophosphatidylcholine (DeRobertis and Vaz Ferreira, 1957; Schneider *et al.*, 1967; Diner, 1967; Douglas, 1968). Since brain neuronal membranes are composed of approximately 70 percent lipid, with the majority of the lipid being phospholipid (Whittaker, 1970), the proposed fusion occurring during exocytosis will necessarily involve lipid interactions. Based on these data, one can predict that the release of NE should be sensitive to the action of phospholipases.

A review of the literature indicates that trypsin is likely to effect NE uptake for the following reasons. (a) Bloom and Aghajanian (1966, 1968) have found that ethanolic phosphotungstic acid (E-PTA) selectively stains the synaptic apparatus when observed under the electron microscope. The reaction of the synaptic material with E-PTA suggests the presence of basic proteins in the synaptic apparatus. This was confirmed by observing that the ability of E-PTA to stain the synaptic material was reduced by prior trypsin digestion. (b) Mild trypsin treatment inactivates the $Na^+$ $K^+$ dependent ATPase often referred to as transport ATPase (Somogyi, 1968). (c) It is presently assumed that the primary locus for NE uptake is the synaptic apparatus and this uptake is $Na^+$ and ATPase dependent (Iversen, 1967; Bogdanski *et al.*, 1970; Tissari and Bogdanski, 1971; White and Keen, 1970). In the present chapter the effects of phospholipases A and C and trypsin on NE uptake and spontaneous release were investigated.

## 8.2 EXPERIMENTAL - CHEMICAL.

The methods for measuring cortical NE uptake and spontaneous release in a crude mitochondrial preparation are described in Chapter 3. These procedures were altered in the following experiments only in that for the uptake experiments the homogenate was preincubated with the various enzymes prior to the addition of $^3$H-NE and in the release experiments the enzymes were added at 0 time in the final incubation. In some experiments NEP isolated from the cortex by the method of Von Hungen *et al.* (1968) were used to measure uptake and release by the same procedure as used for the crude mitochondrial suspension. In addition, NEP treated with the various enzymes were examined by electron microscopy.

The effect of the various enzymes on the protein content of the rat brain homogenate or NEP was analyzed by measuring the protein in the pellet after the washing procedures by the method of Lowry *et al.* (1951). Phospholipids were extracted from the pellet as described by Abdel-Latif and Smith (1970) and the level of total lipid P was determined (Bartlett, 1959). Glycoprotein in the defatted pellet was determined by hydrolyzing the sample for 1 hr in 0.1 N $H_2SO_4$, centrifuging and then analyzing the supernatant for N-acetylneuraminic acid (NANA) by the method of Warren (1959). The activity of phospholipase A was analyzed by pretreating rats with 5 μCi of $^{14}$C-choline (Ch) (specific activity 56 mCi/mmol), preparing a phospholipase A treated pellet as previously described, isolating the phospholipids and determining the conversion of $^{14}$C-lecithin to $^{14}$C-lysolecithin by TLC (Abdel-Latif and Smith, 1970). All of the methods indicated above are described in detail in Chapter 3.

All animals used for uptake and release experiments were pretreated with 75 mg/kg of pargyline i.p. 20 to 24 hr prior to sacrifice.

## 8.3 NE UPTAKE IN RAT BRAIN CORTICAL HOMOGENATE.

The uptake of NE was principally measured in the post-nuclear supernatant as described by Snyder and Coyle (1969). Some characteristics of the uptake of NE in this preparation are illustrated in Fig. 35. These data agree with the findings of others (Bogdanski *et al.*, 1970; White and Keen, 1970; Tissari and Bogdanski, 1971) that the uptake of NE is a $Na^+$ and energy-dependent process which follows Michaelis-Menten kinetics. In our studies, lowering the $[Na^+]$ to 56 mM and making the solution isotonic with 0.32 M sucrose resulted in non-competitive inhibition of NE uptake.

## 8.4 EFFECT OF TRYPSIN, PHOSPHOLIPASE A AND PHOSPHOLIPASE C ON NE UPTAKE.

Using the homogenate preparation, the effects of trypsin, phospholipase A and phospholipase C on NE uptake were investigated. Phospholipase C was found to have no effect on NE uptake despite the 73 percent loss of lipid P (Table 12). Phospholipase A, which caused a 57

Fig. 35. Graphic analysis of the reciprocals of $^3$H-norepinephrine (NE) concentration and its accumulation into a particulate fraction isolated from the rat brain cortex. Homogenates were incubated in duplicate for 5 min with various concentrations of $^3$H-NE. Incubations were performed at 37° C (O), 0-4° C (Δ), and with the ($Na^+$) reduced to 56 mM and the $Na^+$ replaced with 0.32 M sucrose (□). Velocity (V) is expressed as (mμmol of $^3$H/mg protein/5 min) x $10^{-2}$. Control (37° C) - $K_m$ - 1.81 ± 0.17 x $10^{-7}$, $V_{max}$ - 1.00 ± 0.05; low $Na^+$ - $K_m$ 2.46 ± 0.13 x $10^{-7}$, $V_{max}$ - 0.51 ± 0.06; control (0-4° C) - 2.16 ± 0.28 x $10^{-7}$, $V_{max}$ - 0.29 ± 0.06. $K_m$ and $V_{max}$ values were determined by Wilkinson plots and represent the mean ± S.E. of at least 10 determinations.

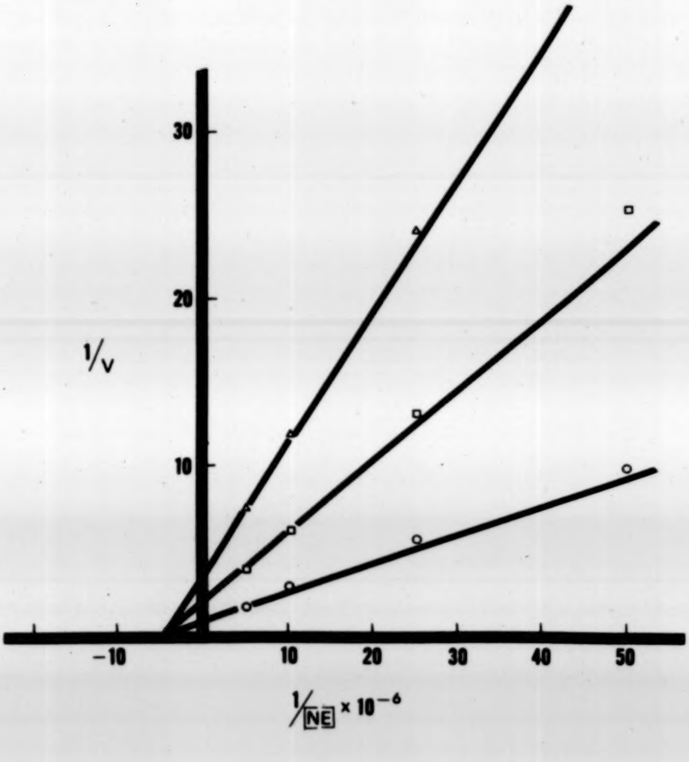

30
20
10
1/v
−10
10
20
30
40
50
1/[NE] × 10⁻⁶

TABLE 12

Effect of Trypsin and Other Enzymes on NE Uptake by Rat Brain Cortical Homogenates [a]

| Enzyme | Enzyme Concentration (μg/ml) | Enzyme Concentration (units/ml) | Min. Incubation | $K_m$ | $V_{max}$ [b] | Percent Change in Pellet Composition [c] Protein | Percent Change in Pellet Composition [c] Phospholipid |
|---|---|---|---|---|---|---|---|
| Trypsin | 200 | 2000 | 45 | $1.94 \pm 0.09 \times 10^{-7}$ | $0.49 \pm 0.02$ | -4 | -2 |
| Phospholipase A | 0.1 | .0004 | 45 | $2.62 \pm 0.32 \times 10^{-7}$ | $0.48 \pm 0.07$ | -33 | * |
| Phospholipase C | 50 | .1 | 45 | $1.92 \pm 0.21 \times 10^{-7}$ | $0.93 \pm 0.06$ | +6 | -73 |
| None | 0 | 0 | 45 | $1.81 \pm 0.17 \times 10^{-7}$ | $1.00 \pm 0.05$ | - | - |

[a] Aliquots of cortical rat brain homogenates diluted in KRB buffer were preincubated at 37° C in the presence of the various enzymes for the times indicated. Control mixtures were incubated without enzyme for 45 min. Various concentrations ($2 \times 10^{-7}$ to $2 \times 10^{-8}$ M) of [$^3$H]-NE were added and uptake was allowed to proceed for 5 min. The mixture was centrifuged for 10 min x 48,000 g and the resulting pellet was washed twice with ice-cold buffer. The pellet was then homogenized in 0.4 N $HClO_4$, centrifuged and a portion of the supernatant was counted for total $^3$H. $K_m$ and $V_{max}$ were determined by Wilkinson plots and the results represent the mean ± S.E. of at least 4 experiments performed in duplicate where 4 amine concentrations were used.

[b] Velocity (V) is expressed as (mμmol/mg protein/5 min) x $10^{-2}$).

[c] The total protein and phospholipid content of the pellet was determined as described in Methods. The percent change from control was calculated from the mean values of 6 determinations.

* In the phospholipase A treated homogenates, there was a conversion of 57% of the total lecithin to lysolecithin. The procedures involved in this determination are outlined in Methods. The percent change was calculated from 6 determinations.

percent conversion of lecithin to lysolecithin and a 33 percent loss of particulate protein, decreased NE uptake non-competitively as illustrated by the significant 52 percent decrease in $V_{max}$ and a non-significant change in $K_m$ (Table 12).

Trypsin, like phospholipase A, was found to decrease NE uptake (Table 12, Fig. 36). However, trypsin treatment caused no detectable change in particulate protein or phospholipid. To determine if the effect of trypsin on uptake was related to proteolytic activity, some additional experiments were performed. The results of these experiments may be summarized: (a) The trypsin effect was concentration and time dependent. The maximum inhibition of NE uptake occurred after 30 to 45 min of preincubation. (b) The trypsin effect was blocked by soybean trypsin inhibitor. (c) Equilibrium dialysis experiments indicated that trypsin does not bind NE. (d) Brain homogenates were incubated with trypsin for 5 and 45 min. The mixture was pelleted, washed twice to remove the trypsin and then used to measure NE uptake. Only in the pellet preincubated for 45 min was NE uptake inhibited. Unless trypsin is absorbed onto the site(s) for uptake in a time dependent fashion, these data indicate that trypsin has produced a permanent alteration of the uptake apparatus.

## 8.5 FURTHER STUDIES ON THE EFFECT OF TRYPSIN ON NE UPTAKE.

Although previous studies (Snyder and Coyle, 1969) have demonstrated that NE uptake in the homogenate preparation occurs almost entirely into the NEP, it seemed necessary to demonstrate that trypsin could decrease NE uptake in a preparation of NEP in order to eliminate the effect of other subcellular structures. The data in Fig. 37 illustrate that prior trypsin treatment decreased NE uptake into NEP. The

Fig. 36. Graphic analysis of the reciprocals of $^3$H-norepinehprine concentration and its accumulation into a particulate fraction isolated from the rat brain cortex showing the effect of prior trypsin digestion (200 μg/ml enzyme x 45 min). Homogenates were incubated in duplicate for 5 min. Velocity (V) is expressed as (mμmol/mg protein/5 min) x $10^{-2}$. Control (●) - $K_m = 1.81 \pm 0.17 \times 10^{-7}$, $V_{max} = 1.00 \pm 0.05$; trypsin (■) - $K_m = 1.94 \pm 0.09 \times 10^{-7}$, $V_{max} = 0.49 \pm 0.02$. $K_m$ and $V_{max}$ values were determined by Wilkinson plots and represent the mean ± S.E. of 10 determinations.

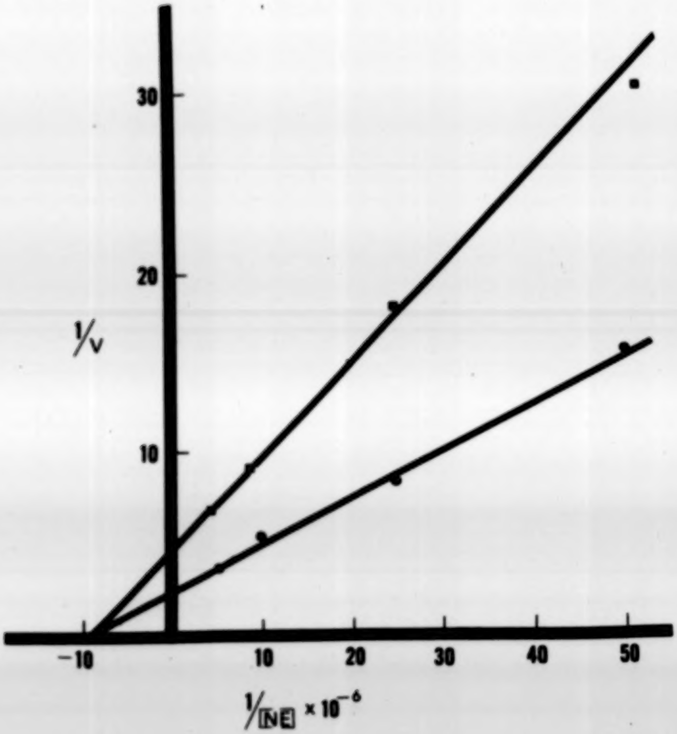
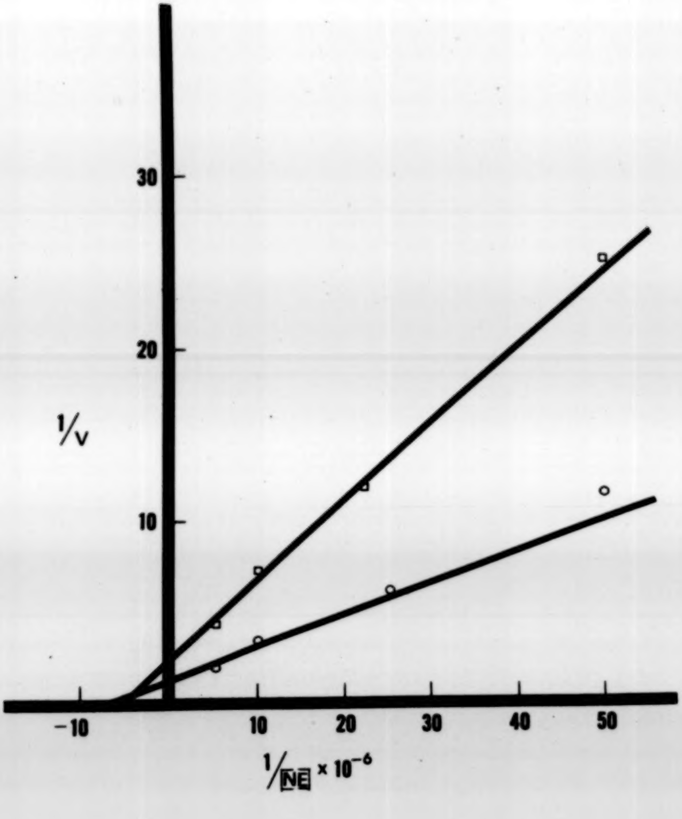

30
20
10
1/v
-10
10
20
30
40
50
1/[NE] × 10⁻⁶

Fig. 37. Graphic analysis of the reciprocals of $^3$H-norepinehprine concentration and its accumulation into nerve ending particles showing the effect of prior trypsin digestion (200 μg enzyme ml x 45 min). Nerve endings were incubated in duplicate with H-norepinephrine for 5 min. Velocity (V) is expressed as mμmol/mg protein/5 min. Control (0) - $K_m = 1.02 \pm 0.11 \times 10^{-7}$, $V_{max} = 0.39 \pm 0.03$; trypsin (□) - $K_m = 1.22 \pm 0.17 \times 10^{-7}$, $V_{max} = 0.21 \pm 0.04$. $K_m$ and $V_{max}$ values were determined by Wilkinson plots and represent the mean ± S.E. of 8 determinations.


30
20
10
1/v
-10
10
20
30
40
50
1/[NE] × 10^-6

decrease in uptake was non-competitive and resulted in a 47 percent decrease in $V_{max}$. Although the incubation time (45 min) used in these studies was fairly long, the viability of the NEP was not greatly altered as evidenced by the fact that NE uptake decreased only 10 to 15 percent over this time period.

The possibility that trypsin treatment decreased only the $Na^+$ stimulated uptake of NE was investigated. The data in Table 13 show that when the external $Na^+$ concentration is lowered to 56 mM, trypsin treatment no longer causes a decrease in uptake.

The data in Table 14 indicate that trypsin did not significantly decrease the uptake of GABA, 5-HT, DA and Ch into a cortical homogenate.

The phenethylamine transport mechanisms in the cortex and caudate nucleus have been suggested to be both similar (Ferris *et al.*, 1972) and dissimilar (Snyder and Coyle, 1969). The data in Table 15 indicate that, as in the cortex, trypsin decreased NE but not DA uptake in the caudate nucleus.

## 8.6 EFFECT OF TRYPSIN ON THE PROTEIN AND GLYCOPROTEIN CONTENT OF NEP.

The data in Table 16 show that trypsin treatment caused a small but significant decrease in the protein content of NEP. Unlike its effect on protein, however, trypsin caused an appreciable loss of glycoprotein as measured by the loss of protein bound N-acetylneuraminic acid (NANA). To some degree, the decrease in glycoprotein correlated with the decrease in NE uptake.

## 8.7 EFFECT OF TRYPSIN ON THE MORPHOLOGY OF NEP.

Representative electron micrographs of control, trypsin and phos-

## TABLE 13

### Effect of Trypsin on Norepinephrine Uptake in a Low Na$^+$ Buffer [a]

| Group | (Na$^+$)<br>140 mM | 56 mM |
|---|---|---|
| Control | 5.19 ± 0.31 | 2.91 ± 0.33 |
| Trypsin | 3.60 ± 0.24* | 2.84 ± 0.37 |

[a] Aliquots of a cortical homogenate were rediluted in normal KRB buffer or in a buffer when the Na$^+$ had been partially replaced by 0.32 M sucrose. The samples were preincubated with trypsin (200 μg/ml x 15 min) prior to the addition of $10^{-7}$ M [$^3$H]-NE. Uptake was terminated after 5 min and the total $^3$H in a particulate fraction was determined as previously described. Data are expressed as (mean mμmol $^3$H/mg protein/5 min ± S.E.) x $10^{-3}$ and is the result of 5 determinations performed in duplicate.

* Significantly different than control - $p < 0.05$.

### TABLE 14

### Effect of Trypsin on the Uptake of 5-HT, Ch, GABA and DA [a]

| Group | Control | Trypsin |
|---|---|---|
| | mμmol/g/5 min x $10^{-2}$ | |
| 5-HT ($2 \times 10^{-7}$ M) | 0.12 ± 0.01 | 0.10 ± 0.02 |
| GABA ($1 \times 10^{-5}$ M) | 10.21 ± 0.34 | 10.98 ± 0.79 |
| Ch ($1 \times 10^{-7}$ M) | 3.13 ± 0.42 | 3.42 ± 0.58 |
| DA ($1 \times 10^{-7}$ M) | 0.51 ± 0.04 | 0.49 ± 0.06 |

[a] Aliquots of a homogenate prepared from the cortex were diluted in KRB buffer and preincubated for 45 min in the presence of 200 μg/ml trypsin. After preincubation, the various putative neurotransmitters were added to give the final concentrations indicated and uptake was allowed to proceed for 5 min. Total $^3H$ in a particulate fraction of the homogenate was determined as described. The data are presented as the mean ± S.E. of four determinations performed in duplicate.

### TABLE 15

### Effect of Trypsin on the Uptake of NE and DA in the Caudate Nucleus [a]

| Group | NE | DA |
|---|---|---|
| | (mμmol/mg protein/5 min) $\times 10^{-2}$ | |
| Control | 1.83 ± 0.11 | 3.12 ± 0.25 |
| Trypsin | 1.02 ± 0.12* | 2.87 ± 0.19 |

[a] Aliquots of a homogenate prepared from the caudate nucleus were diluted in KRB buffer and preincubated for 45 min in the presence of 200 μg/ml trypsin. After preincubation, $4 \times 10^{-7}$ M [$^3$H]-NE or [$^3$H]-DA was added and uptake was allowed to proceed for 5 min. Total $^3$H in a particulate fraction of the homogenate was determined as described. The data are presented as the mean ± S.E. of 5 determinations performed in duplicate.

* Significantly different than control - $p < 0.05$

## TABLE 16

### Effect of Trypsin on NEP Protein, Glycoprotein and NE Uptake [a]

| Enzyme Concentration (μg/ml) | mg protein/pellet ± S.E. | μg NANA/mg protein ± S.E. | NE uptake percent change ± S.E. |
|---|---|---|---|
| 0 | 3.93 ± 0.06 | 3.63 ± 0.13 | - |
| 50 | 3.87 ± 0.04 | 3.18 ± 0.06* | -18 ± 2* |
| 100 | 3.73 ± 0.03* | 2.89 ± 0.08* | -29 ± 3* |
| 200 | 3.72 ± 0.03* | 2.75 ± 0.03* | -41 ± 5* |

[a] NEP were incubated in 5 ml of KRB buffer containing various concentrations of trypsin for 45 min at 37° C prior to the addition of $[^3H]$-NE $-2 \times 10^{-7}$ M. After 5 min, uptake was terminated by centrifugation for 20 min x 50,000 g. The total protein, glycoprotein and $^3H$ in the pellet was determined as described in the Methods. Glycoprotein data are expressed as μg of glycoprotein NANA per mg of total protein.

* Significantly different than control - $p < 0.05$.

pholipase C treated NEP are shown in Fig. 38-40. No gross morphological alterations were observed in the trypsin treated NEP using the staining procedures described in the Methods. However, the phospholipase C treated NEP typically appeared hazy and poorly stained. In addition, qualitatively there was greater disorganization of the NEP mitochondrial cristae.

## 8.8 EFFECT OF VARIOUS ENZYMES ON SPONTANEOUS RELEASE.

The spontaneous release or efflux of NE from NEP in the crude mitochondrial fraction was temperature dependent and to some degree could be stimulated by increasing the $K^+$ concentration to 50 mM (Fig. 41). All three enzymes enhanced spontaneous release with the apparent order of potency being phospholipase A > phospholipase C > trypsin (see Table 12 for units of enzyme activity in Fig. 41). The concentrations of enzyme used are those in Tables 12 and 16 where information on hydrolysis of membrane components appear. The effects of trypsin and phospholipase C but not phospholipase A on efflux could be stopped by immersing the reaction vesicle in ice-water (Fig. 42). One explanation of these data is that phospholipase A has rendered the NEP "leaky" while phospholipase C and trypsin have affected the release process per se, although other explanations are certainly possible.

## 8.9 DISCUSSION.

The data described in this chapter contain two findings which may be of some importance. (a) Trypsin treatment specifically decreases NE uptake into NEP; the uptake of DA, 5-HT or Ch is not affected. (b) Phospholipase C treatment enhances NE efflux but has no effect on reuptake. Preliminary data indicate that, unlike trypsin, the phospho-

Fig. 38. Electron micrographs of some typical control NEP prepared from rat brain cortical tissue. NEP prepared from the rat cortex were resuspended in KRB and incubated in the presence of trypsin, phospholipase C or no enzyme. Incubation conditions are further described in the legend to Table 12. After incubation, the NEP were pelleted, fixed and prepared for electron microscopy as described in Section 3.4. Micrograph pictures were randomly taken in a single-blind fashion by the EM technician. Magnification x 25,000.

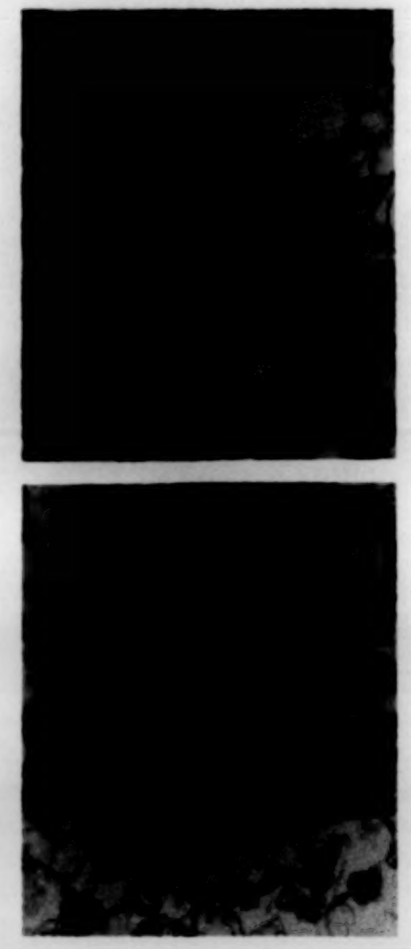


Fig. 39. Electron micrographs of trypsin treated NEP from the rat brain cortex. Details are given in the legend to Fig. 38. The trypsin to NEP protein ratio was 1 to 5.

XUM

Fig. 40. Electron micrographs of phospholipase C treated NEP prepared from the rat brain cortex. The phospholipase C to NEP protein ratio was 1 to 20. Details are given in the legend to Fig. 38.

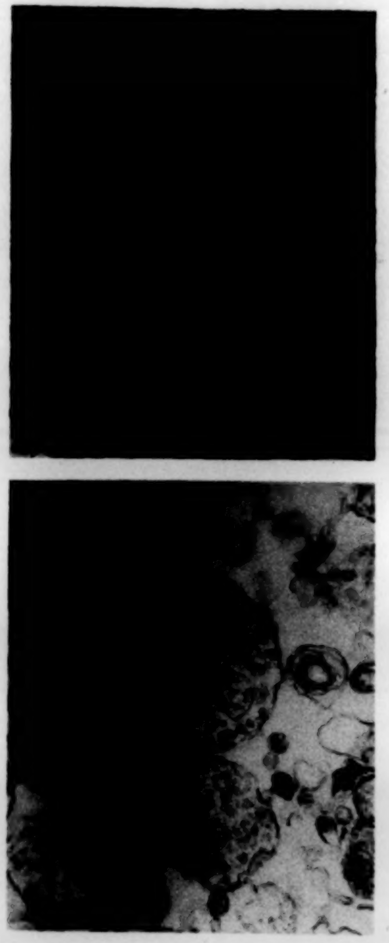
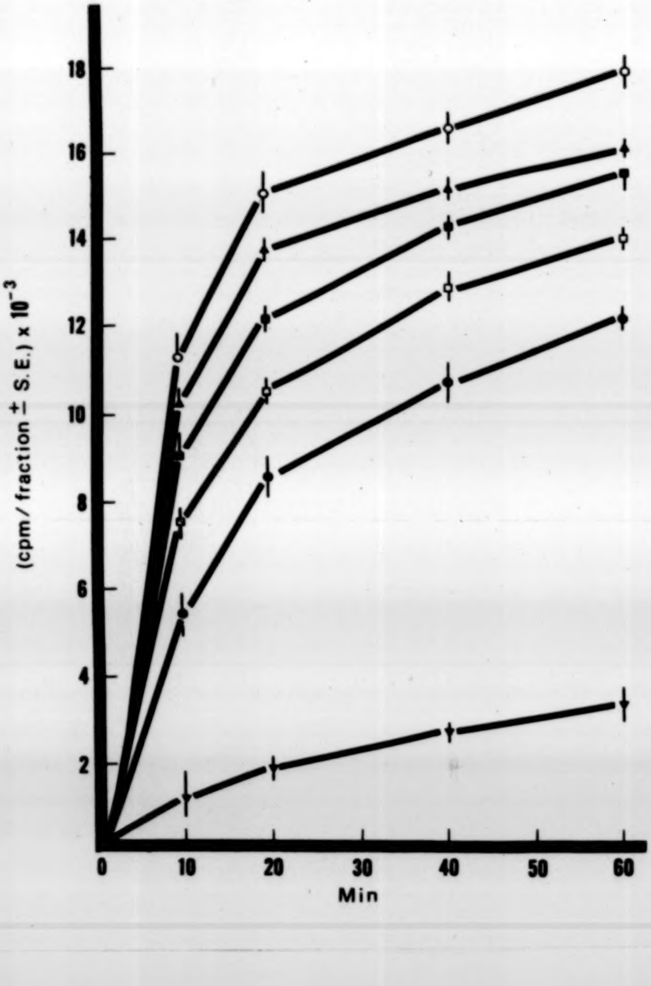


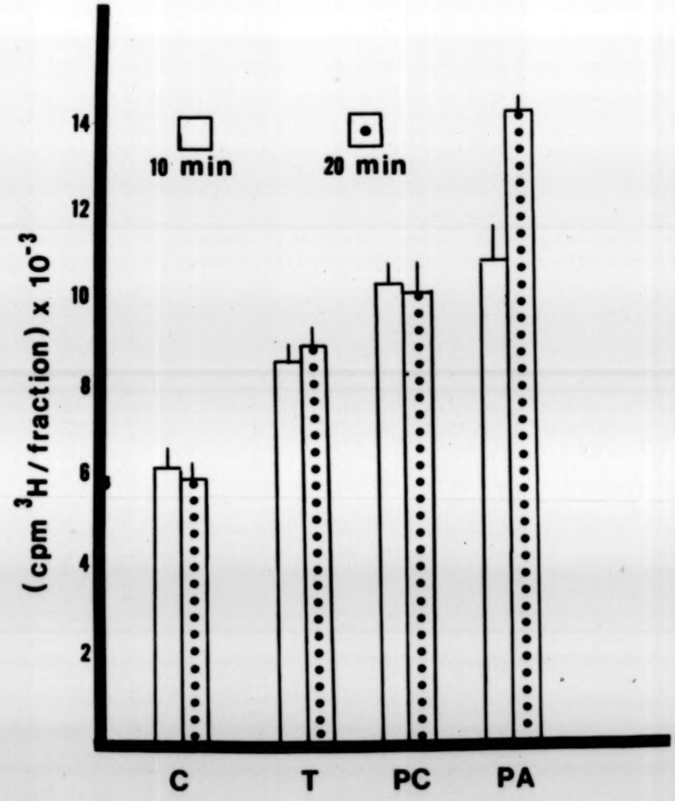
Fig. 41. Rate of $^3$H-norepinephrine efflux from a particulate fraction of the rat brain cortex showing the effect of the additions of trypsin, 200 µg/ml (■), phospholipase C, 50 µg/ml (▲), phospholipase A, .1 µg/ml (0) and 50 mM $K^+$ ( ), and the effect of incubations performed at 0-4° C (▼). Control incubations (●) were performed at 37° C. Homogenates were incubated in the presence of $10^{-7}$ M $^3$H-norepinephrine for 10 min and then centrifuged for 15 min x 48,000 g. The pellet was washed several times and finally resuspended in ice-cold Krebs buffer which in some experiments contained trypsin or phospholipases A or C. The mixtures were incubated at 37° or 0° C for 1 hr. One ml aliquots of the reaction mixture were removed at various times, centrifuged for 10 min x 48,000 g and 0.2 ml of the supernatant was analyzed for total $^3$H. Each point represents the mean ± S.E. of 10 experiments performed in duplicate.

XUM



(cpm / fraction ± S.E.) x $10^{-3}$
18
16
14
12
10
8
6
4
2
0
10
20
30
40
50
60
Min

Fig. 42. Effect of temperature on the enzyme-induced alterations in the spontaneous release of NE from a particulate fraction of the rat brain cortex. This experiment was performed exactly as those described in the legend to Fig. 41 except that 10 min after the addition of enzymes, the reaction vesicles were immersed in an ice-water bath. Release was then measured at 10 and 20 min after this manipulation. N = mean ± S.E. of six experiments performed in duplicate. Legend: C = Control; T = Trypsin; PC = Phospholipase C; PA = Phospholipase A.


(cpm $^3$H / fraction) x 10$^{-3}$
14
12
10
8
6
4
2
10 min
20 min
C
T
PC
PA

lipase C effect is not amine specific. The relevance of each of these findings will be discussed in some detail.

When examining the effect of trypsin on membrane components and reuptake, it is perhaps easier to state what trypsin apparently does not do. (a) Trypsin had no appreciable effect on the total protein and, incidentally, phospholipid content of the brain homogenate or the NEP. (b) Preliminary experiments indicate that trypsin does not alter the gross morphology of the NEP. (c) The decrease in NE uptake caused by trypsin is apparently not related to the increase in NE efflux for two reasons. One, by using a short incubation time (5 min), the effect of efflux on the level of NE taken up into the NEP is probably small. Two, phospholipase C, which caused an increase in efflux, had no effect on uptake.

It appears that the principal effect of trypsin on NEP is to decrease the glycoprotein content. Cytochemical and biochemical studies have shown that the outer surface of the neuron is covered with glycoproteins and glycolipids (Schmitt and Samson, 1969; Rambourg and Leblond, 1967). Assuming only a non-significant amount of trypsin is taken inside the NEP, it would appear that trypsin is "stripping" off superficial glycoprotein. This conclusion is supported by the observations of Carraway *et al.* (1971). These authors have found that extensive trypsin digestion of intact human erythrocytes produces no change in the erythrocyte membrane proteins as judged by disc gel electrophoresis. However, external membrane glycoproteins were accessible and susceptible to trypsin-induced hydrolysis.

Neuronal membrane glycoproteins are thought to have some role in development (Brunngraber, 1969; Dische, 1966), information storage

(Brunngraber, 1969b, Brunngraber, 1970; Bogoch, 1968) and in ion movements (Schmitt and Samson, 1969). The fixed anions of neuronal glycoproteins or glycolipids which extend into the external milieu can bind with monovalent or divalent cations; this binding leads to a conformational change of the carbohydrate moiety of the glycoprotein which, in turn, may cause conformational changes in other portions of the neuronal membrane (Schmitt and Samson, 1969). The binding of cations to glycoproteins may be of some importance in the present study since it was observed that lowering the $Na^+$ concentration blocked the trypsin effect on NE uptake. Perhaps the mechanism by which trypsin inhibits NE uptake involves a decrease in the $Na^+$ binding to a membrane glycoprotein component required for NE transport. This conclusion is, however, quite speculative and other interpretations are possible. For example, NE uptake at high and low $Na^+$ concentrations may occur by more than one transport mechanism, with only the high $Na^+$ concentration mechanism being sensitive to trypsin. If superficial glycoproteins are involved in NE uptake, there would seem to be two ways to test this hypothesis. One, enzymes which specifically attack various carbohydrate moieties of glycoproteins may alter NE uptake. In this regard, I have found that the incubation of NEP with a crude commercial neuraminidase preparation has no effect on uptake (preliminary observation). Two, the effect of plant lectins on NE uptake may prove to be a valuable tool in elucidating the role of glycoproteins. Gombos *et al.* (1972) have observed that concanavalin A agglutinates NEP and this agglutination can be reversed by α-methylmannoside. Peters and Hausen (1971) have observed that a phytohemagglutinen increases the uptake of 3-0-methyl-glucose into lymphocytes.

The lack of trypsin effect on DA uptake deserves some special comment. Snyder and Coyle (1969) have suggested that in the range of amine concentrations employed in the present study, DA uptake in the cortex primarily occurs via a NE carrier while NE uptake in the caudate nucleus occurs primarily via a non-stereospecific DA carrier. However, in both brain regions, trypsin treatment affected only NE and not DA uptake. From these data, it is tempting to speculate that the differences in the amine pumps of the cortex and the caudate nucleus are not as great as originally predicted by Snyder and Coyle (1969). Recently, Ferris *et al.* (1972) have presented evidence indicating that the phenethylamine pumps of the cortex and striatum have many similarities. The differentiation of the two transport system on the basis of the effects of the tricyclic antidepressants (Snyder and Coyle, 1969) appears to be related to the fact that the striatal system cannot accept the fixed ring structure of these drugs (Ferris *et al.*, 1972). Overall, the data of Ferris *et al.* would suggest that an alteration in binding occuring at the β-OH site may not be elucidated by comparing the uptake of NE and DA in the striatum. Thus, the possibility that such an alteration occurs after trypsin treatment must still be considered as a mechanism of the enzyme effect.

The ability of trypsin treatment to block NE uptake is analogous to the findings of others in regard to the inhibitory effect of trypsin on the activity of some biological systems. Somogyi (1968) has found that digestion of brain microsomes with trypsin but not α-chymotrypsin or substillisin A decreases the activity of the $Na^+$, $K^+$-activated ATPase under experimental conditions where the latter enzymes produced a greater rate of proteolytic digestion. Sellinger *et al.* (1969) have found that trypsin, but not neuraminidase, inactivated synaptosomal acetylcholinesterase. Tomasi *et al.* (1970) have reported that trypsin treatment blocks the binding of epinephrine to a receptor protein in hepatic membrane preparations.

If it is possible to extrapolate from the red blood cell ghost to neuronal membranes, then the arrangement of phospholipids in neuronal membranes is probably asymmetric; phosphatidylcholine is located primarily on the exterior surface of the lipid bilayer and phosphatidylethanolamine and ethanolamine plasmologen are located interior (Bretscher, 1973). The minor phospholipids, in terms of content, phosphatidylserine and the phosphatidylinositols may or may not be "randomly" distributed throughout the membrane. Assuming phosphatidylcholine is located externally provides an important theoretical viewpoint from which the effect of phospholipase C on NE reuptake and release can be examined. The fixed positive charge of phosphatidylcholine may serve as a barrier preventing other positively charged molecules such as cations and weak bases (at physiological pH) from crossing the nerve ending membrane. However, this does not seem to be true in regard to NE transport. The data in Table 15 clearly demonstrate that even severe phospholipase C treatment had no effect on uptake. Since there is no evidence that phospholipase C penetrates inside the nerve ending, it is probably safe to assume that enzyme primarily hydrolyzes phosphatidylcholine. While removal of the phosphorylcholine moiety may have no effect on NE uptake, this treatment is not without some influence on cell structure (Fig. 37). In normal erythrocytes, Bowman *et al.* (1971) have observed that phospholipase C causes cell shrinking, sphering, increased susceptibility to osmotic stress and progressive homolysis.

Based on the data illustrated in Fig. 41, it can be seen that phospholipase C treatment has a marked effect on the spontaneous release of NE; preliminary studies suggest that the phospholipase C effect is not related to the production of "leaky" nerve endings, but rather is specific for the energy dependent outward transport of NE. The unanswerable question is to what degree is the efflux measured in Fig. 41 similar to normal release? At this time it is not possible to argue pro or con on this point. The value of these data is that we can predict that pharmacologic or other influences which functionally mimics the phospholipase C effect should increase spontaneous release. The question then arises, must these influences actually result in the loss of phosphorylcholine from the membrane? I think not. Rather, it is proposed that if the turnover of the phosphorylcholine moiety is decreased, then release should increase, all other factors being equal. It is this hypothesis that prompted the investigations of the effects of *d*-amphetamine on phosphatidylcholine synthesis and turnover described in Chapters 9 and 10.

Phospholipase A probably caused the decrease in NE reuptake and increased spontaneous release by breaking the nerve ending plasma membrane, although an effect of the increase in free fatty acid levels cannot be excluded. Heilbronn and Cedergren (1970) and Cedergren *et al.* (1970) have shown by morphological analysis that phospholipase A is able to break and digest the external membrane of nerve ending particles but leaves the junctional apparatus intact. It is interesting to note that the effectiveness of phospholipase A implies that the fatty acid tails of the phospholipids are not buried in the membrane but rather are accessible to enzyme action.

# CHAPTER 9

# EFFECT OF *d*-AMPHETAMINE ON THE TURNOVER, SYNTHESIS AND METABOLISM OF BRAIN PHOSPHATIDYLCHOLINE

## 9.1 INTRODUCTION.

Based on the data and conclusions presented in Chapters 7 and 8, it can be postulated that one mechanism by which chronic amphetamine intoxication enhances NE release would be to decrease brain phosphatidylcholine turnover. The hypothesis that amphetamine alters brain phospholipid metabolism is strengthened by the observations that DA or NE affects the synthesis of these complex lipids. Hokin (1969, 1970), using a brain tissue slice preparation, has found that NE increases the incorporation of $^{32}P$ into phosphatidic acid and in some brain regions phosphatidylinositol, but decreases $^{32}P$ incorporation into phosphatidylcholine and phosphatidylethanolamine plus phosphatidylserine. Similarly, DA decreased $^{32}P$ incorporation into phosphatidylcholine in slices prepared from the neostriatum. Thus, catecholamines influence phosphatidylcholine synthesis in the same direction that was postulated for amphetamine.

Alterations in phosphatidylcholine synthesis may result in changes in catecholamine transport for several additional reasons not yet discussed. Phosphatidylcholine can protect $Na^+$, $K^+$-stimulated adenosine triphosphatase and mitochondrial adenosine triphosphatase against inhibition by oligomycin (Bruni *et al.*, 1971). These data suggest that phosphatidylcholine may play a regulatory role in ATPase activity. Lysophosphatidylcholine has been postulated to have an important role in the release of catecholamines from the adrenal medulla (Smith, 1968). Finally, since phosphatidylcholine is the major complex lipid component of nerve ending and storage vesicle membranes (Whittaker, 1969), any change in the synthesis or metabolism of this substance could be expected to alter nerve ending integrity, structure or function. In the studies described in this chapter, the effect of chronic *d*-amphetamine on the turnover, synthesis and metabolism of $^{14}$C-choline labeled phosphatidylcholine was investigated in discrete regions of the rat brain.

## 9.2 EXPERIMENTAL - CHEMICAL.

The effect of chronic *d*-amphetamine administration on the turnover of $^{14}$C-choline labeled phosphatidylcholine was examined as follows. Male Sprague-Dawley rats weighing 150 g (age 60-70 days) were given 5 μCi $^{14}$C-choline i.cist. Five days later twice daily injections (i.p.) of 1 mg/kg of *d*-amphetamine or saline were begun. The dose of *d*-amphetamine was doubled every 5 days with the experiment terminating 15 days after the first *d*-amphetamine injection. Animals were sacrificed 15 hr after their last injection on days 10, 15 and 20. The control groups sacrificed on day 5 received no drug treatment. The brains were dissected into the following regions: hypothalamus, caudate nucleus, brain stem, cortex, cerebellum, and diencephalon-midbrain. The dissected tissue was immediately homogenized in a Teflon-glass homogenizer with 9 vol of 0.32 M sucrose containing 1 mM $MgCl_2$ and 1 mM Tris buffer, pH = 6.8. The homogenate was then fractionated into crude mitochondrial, microsomal and soluble fractions as described in Section 3.4. All fractions were frozen at -10° C until analysis for phosphatidylcholine as described in Section 3.5.

The effect of acute *d*-amphetamine treatment on the incorporation of $^{14}$C-choline into phosphatidylcholine was determined as follows. Animals were given *d*-amphetamine i.p. 0.5 hr prior to the injection of 5 µCi $^{14}$C-choline i.cist. One-half hr later the animals were sacrificed, brains removed and dissected and immediately homogenized in 10 percent cold TCA. The homogenate was centrifuged for 10 min at 5000 g. The pellet was washed twice with 5 percent trichloroacetic acid (TCA) containing 1 percent choline prior to analysis for phospholipids. The first TCA extract was analyzed for $^{14}$C-choline as described in Section 3.6.

In some experiments, tissue slices (0.26 mm) prepared in KRB buffer (Katz and Kopin, 1967) were incubated with 5 µCi of $^{14}$C-choline with or without various concentrations of *d*-amphetamine. The experiment was terminated by the addition of sufficient TCA to bring the final concentration to 6 percent and followed by rapid homogenization. The homogenate was processed as described for the acute *in vivo* experiments. The TCA extract from these studies was analyzed for $^{14}$C-choline and $^{14}$C-choline metabolites as described in Section 3.6.

## 9.3 TURNOVER OF $^{14}$C-PHOSPHATIDYLCHOLINE IN DISCRETE BRAIN REGIONS.

Thin-layer chromatographic analysis of the phospholipid extract from the various brain regions demonstrated that more than 95 percent of the total activity was located in $^{14}$C-phosphatidylcholine from day 5 to day 20. The small amount of radioactivity present as $^{14}$C-lysophosphatidylcholine or $^{14}$C-sphingomyelin prevented the accurate determination of the turnover of these compounds. Also, the amount of $^{14}$C-phosphatidylcholine in the soluble fraction was found in less than trace amounts. The level of unincorporated $^{14}$C-label in the soluble fraction was negligible (< 50 dpm/mg sol protein) by day 5 when the experimental period began. It was considered that with such a low level of radioactivity any drug-induced changes in free $^{14}$C-choline specific activity would not greatly affect the results.

The control turnover (or disappearance) of $^{14}$C-phosphatidylcholine generally shows a half-life value ($t_{\frac{1}{2}}$) of 1 to 2 weeks for the crude mitochondrial and microsomal fractions in the various brain regions (Table 17). An accurate determination of a true $t_{\frac{1}{2}}$ value would require that the experimental period last at least three half-lives or, in some cases in the present study, 60 days or more. Thus, the data obtained in this study where an experimental interval of 15 days was used is useful only for comparing turnover in normal and drug treated groups.

Figures 43 and 44 illustrate that the control turnover of $^{14}$C-phosphatidylcholine showed considerable variance in rank order between the mitochondrial and microsomal fractions (see Table 17). In the mitochondrial fraction, the order of decreasing rate of $^{14}$C-phosphatidylcholine turnover was brain stem (14 days), cerebellum (14 days), diencephalon (16 days), hypothalamus (18 days), cortex (21 days) and caudate nucleus (23 days). The order of decreasing rate of $^{14}$C-phosphatidylcholine turnover in the microsomal fraction was hypothalamus (6 days), caudate nucleus (7 days), cerebellum (7 days), brain stem (8 days), diencephalon (12 days) and cortex (17 days).

## 9.4 EFFECT OF CHRONIC *d*-AMPHETAMINE TREATMENT ON $^{14}$C-PHOSPHATIDYLCHOLINE TURNOVER.

Beginning 5 days after $^{14}$C-choline administration, animals were given *d*-amphetamine using a progressively increasing dosage schedule. *d*-Amphetamine was given in doses of 1, 2 and 4 mg/kg with the dose

## TABLE 17

## Effect of Chronic *d*-Amphetamine Treatment on the Turnover of $^{14}$C-Phosphatidylcholine [a]

| | $T_{1/2}$ (days) | | | |
|---|---|---|---|---|
| | Mitochondrial | | Microsomes | |
| Brain Region | Control | Amphetamine | Control | Amphetamine |
| Cortex | 21 | 30* | 17 | 23* |
| Cerebellum | 14 | 17 | 7 | 10 |
| Brain Stem | 14 | 29* | 8 | 15* |
| Diencephalon | 16 | 25* | 12 | 25* |
| Caudate nucleus | 23 | 46* | 6 | 8 |
| Hypothalamus | 18 | 24* | 6 | 8 |

[a] Animals were given 5 µCi $^{14}$C-choline i.cist. dissolved in 20 µl of saline. Twice daily injections of *d*-amphetamine were begun 5 days later and continued for 15 days. Injections were begun at 1 mg/kg i.p. and doubled every 5 days. Animals were sacrificed at various times after beginning amphetamine injection and the levels of $^{14}$C-phosphatidylcholine were determined in the crude mitochondrial and microsomal pellets prepared from the various brain regions. Half-life values ($T_{1/2}$) were determined by linear regression.

* Significantly different from control, $p < 0.05$. Significance was determined as described in the text.

Fig. 43. Turnover of $^{14}C$-phosphatidylcholine in the crude mitochondrial fraction of discrete regions of the rat brain in normal (●) and chronic amphetamine-treated (▲) animals. Animals were given 5 μCi $^{14}C$-choline (spec. act. 54 mCi/m-mole) i.cist. Beginning 5 days after $^{14}C$-choline administration, animals were given 1 mg/kg of *d*-amphetamine i.p. twice daily. The dose of *d*-amphetamine was doubled 5 days later and doubled once again 10 days later. Control groups were sacrificed on days 5, 10, 15 and 20. Amphetamine-treated groups were sacrificed 12 hr after their last injection on days 10, 15 and 20. Data are plotted as the log dis./min/micromole of lipid P ± S.E. vs. time. Each point represents the mean of six determinations performed in duplicate.

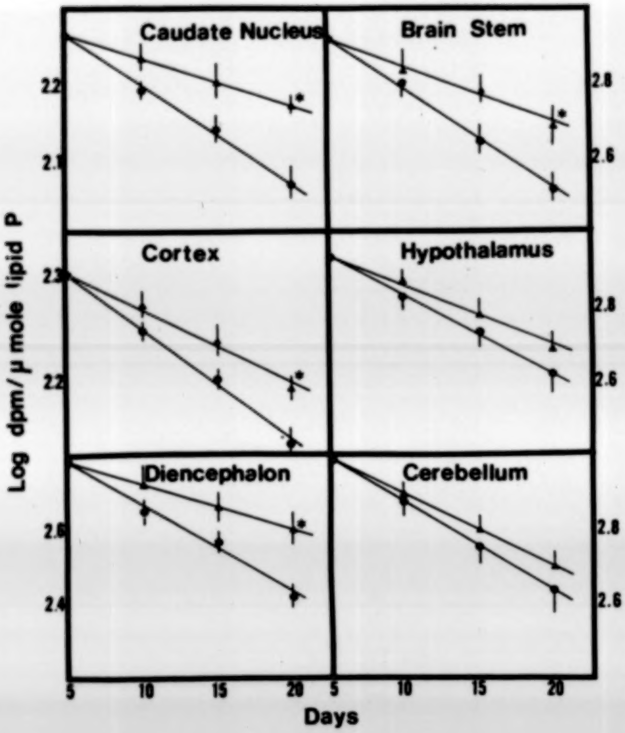
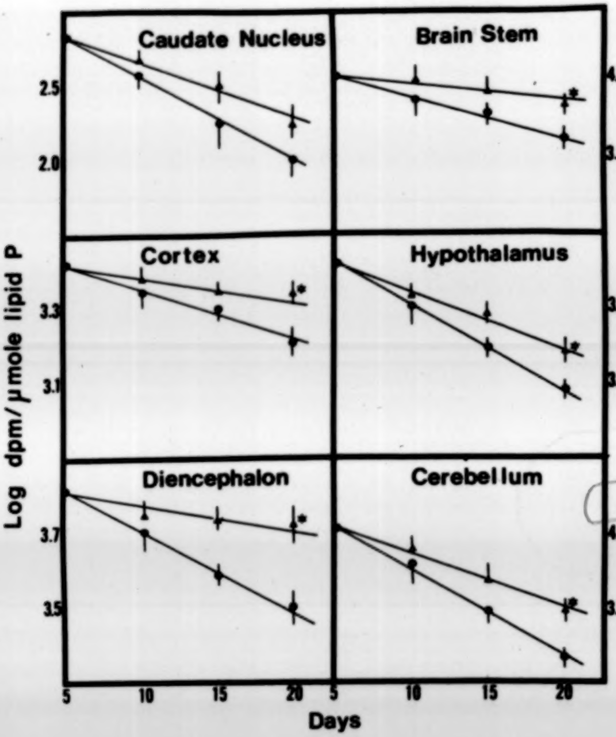

Caudate Nucleus
Brain Stem
Cortex
Hypothalamus
Diencephalon
Cerebellum
Log dpm/μ mole lipid P
Days
2.2
2.1
2.3
2.2
2.6
2.4
2.8
2.6
2.8
2.6
2.8
2.6
5
10
15
20
5
10
15
20
*
*
*
*

Fig. 44. Turnover of $^{14}$C-phosphatidylcholine in the microsomal fraction of discrete regions of the rat brain in normal (●) and chronic amphetamine-treated (▲) animals. Details are described in the legend to Fig. 40.


Caudate Nucleus
Brain Stem
Cortex
Hypothalamus
Diencephalon
Cerebellum
2.5
2.0
4.0
3.5
3.3
3.1
3.5
3.0
3.7
3.5
4.0
3.5
*
*
*
*
*
5
10
15
20
5
10
15
20
Days
Log dpm/μmole lipid P

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being doubled every 5 days. Significant changes in turnover were determined to have occurred when the specific activity on day 20 was significantly different from control. Chronic *d*-amphetamine treatment generally caused a decrease in $^{14}$C-phosphatidylcholine turnover in both the crude mitochondrial and microsomal fractions (Fig. 43 and 44 and Table 17). In the mitochondrial fraction, the caudate nucleus, cortex and brain stem showed the greatest inhibition of turnover, while the cerebellum was least affected. *d*-Amphetamine was found less effective in altering $^{14}$C-phosphatidylcholine turnover in the microsomal fraction as compared to the mitochondrial fraction. In the microsomal fraction the turnover rates of $^{14}$C-phosphatidylcholine in the brain stem and diencephalon were most affected, while the turnover rates in the hypothalamus, caudate nucleus and the cerebellum were only slightly affected by *d*-amphetamine treatment.

## 9.5 EFFECT OF ACUTE *d*-AMPHETAMINE TREATMENT ON $^{14}$C-PHOSPHATIDYLCHOLINE SYNTHESIS.

Animals were given 1 or 10 mg/kg of *d*-amphetamine i.p. 0.5 hr prior to the injection of 5 μCi $^{14}$C-choline i.cist. After 0.5 hr the animals were sacrificed and the levels of $^{14}$C-choline and $^{14}$C-phosphatidylcholine determined. The data in Table 18 show that *d*-amphetamine significantly inhibited the incorporation of $^{14}$C-choline into phosphatidylcholine in the cortex and cerebellum. In other brain regions, notably the hypothalamus and diencephalon, a slight stimulation of synthesis was observed.

TABLE 18

Effect of Acute *d*-Amphetamine Treatment on the Synthesis of $^{14}$C-Phosphatidylcholine [a]

| Brain Region | Dose of *d*-Amphetamine (mg/kg) | $^{14}$C-Choline (dpm/g ± S.E.) x $10^{-5}$ | $^{14}$C-Phosphatidylcholine (dpm/μmol lipid P ± S.E.) x $10^{-1}$ |
|---|---|---|---|
| Cortex | 0 | 11.6 ± 3.7 | 25.8 ± 3.9 |
| | 1 | 12.7 ± 4.2 | 19.0 ± 1.6 |
| | 10 | 10.8 ± 3.1 | 15.0 ± 1.8* |
| Cerebellum | 0 | 104.0 ± 20.2 | 187.0 ± 28.2 |
| | 1 | 103.0 ± 18.7 | 109.0 ± 7.3* |
| | 10 | 91.8 ± 13.4 | 39.8 ± 1.7* |
| Hypothalamus | 0 | 8.3 ± 2.2 | 39.2 ± 3.5 |
| | 1 | 4.5 ± 1.1 | 35.1 ± 2.4 |
| | 10 | 7.1 ± 1.8 | 48.6 ± 3.6 |
| Caudate Nucleus | 0 | 7.1 ± 1.3 | 10.3 ± 2.6 |
| | 1 | 7.3 ± 1.4 | 11.3 ± 3.6 |
| | 10 | 8.4 ± 1.7 | 8.6 ± 1.4 |
| Brain Stem | 0 | 114.0 ± 15.7 | 35.2 ± 4.1 |
| | 1 | 142.0 ± 14.1 | 38.0 ± 4.1 |
| | 10 | 85.3 ± 10.2 | 26.8 ± 6.8 |
| Diencephalon | 0 | 36.3 ± 5.2 | 6.3 ± 2.4 |
| | 1 | 36.6 ± 9.9 | 13.9 ± 4.2 |
| | 10 | 27.3 ± 8.2 | 7.2 ± 1.6 |

[a] Rats were administered *d*-amphetamine i.p. 0.5 hr prior to the injection of 5 μCi of $^{14}$C-choline (specific activity 54 mCi/mmol). 0.5 hr later the animals were sacrificed and the levels of $^{14}$C-choline and $^{14}$C-phosphatidylcholine were determined in discrete brain regions. N = 6 animals per drug dose. Data taken from Hitzemann and Loh (1973).

* Significantly different than control, $p < 0.05$.

## 9.6 EFFECT OF *d*-AMPHETAMINE ON THE INCORPORATION OF $^{14}$C-CHOLINE INTO PHOSPHATIDYLCHOLINE *In vitro*.

The effect of *d*-amphetamine on the incorporation of $^{14}$C-choline into phosphatidylcholine in cortical tissue slices was investigated. The data in Table 19 demonstrate that $10^{-3}$ M *d*-amphetamine had no effect on the incorporation of $^{14}$C-choline into phosphatidylcholine and $10^{-2}$ M decreased incorporation 23 percent ($p < 0.05$). Pretreatment of animals with 10 mg/kg of *d*-amphetamine i.p. 0.5 hr prior to sacrifice resulted in a 39 percent decrease in $^{14}$C-choline incorporation.

Different from the tissue slice experiments, *d*-amphetamine did not alter the formation of $^{14}$C-phosphatidylcholine in a cortical brain homogenate. Additions *in vitro* of *d*-amphetamine ($10^{-4}$ - $10^{-2}$ M) or pretreatment with 10 mg/kg of drug i.p. had no apparent effect on the incorporation of $^{14}$C-choline into phosphatidylcholine (Table 20).

## 9.7 EFFECT OF *d*-AMPHETAMINE ON $^{14}$C-CHOLINE METABOLISM.

The TCA-soluble radioactivity (Table 19) from the tissue slice experiments was analyzed for $^{14}$C-choline metabolites. At $10^{-2}$ M *d*-amphetamine, the levels of $^{14}$C-phosphorylcholine were unchanged but the levels of $^{14}$C-cytidine diphosphorylcholine were more than doubled. In animals pretreated with 10 mg/kg of *d*-amphetamine, a small decrease in $^{14}$C-phosphorylcholine was observed but a significant increase in $^{14}$C-cytidine diphosphorylcholine levels was also found.

## 9.8 EFFECT OF *d*-AMPHETAMINE ON BASE-EXCHANGE NONENZYMATIC INCORPORATION.

Several workers have demonstrated that serine, ethanolamine and choline may be incorporated into phospholipids via a $Ca^{++}$-stimulated

## TABLE 19

### Effect of *d*-Amphetamine on the Incorporation of $^{14}C$-Choline Into Phosphatidylcholine in Cortical Slices *

| Group | $^{14}C$-phosphatidylcholine (dpm/μmole lipid P ± S.E.) | $^{14}C$-Ch (dpm/g ± S.E. x $10^{-5}$) | $^{14}C$-PCh (dpm/g ± S.E. x $10^{-5}$) | $^{14}C$-CDPCh (dpm/g ± S.E. x $10^{-5}$) |
|---|---|---|---|---|
| Control | 6100 ± 470 | 23.79 ± 1.82 | 2.23 ± 0.15 | 0.97 ± 0.07 |
| Amphetamine ($10^{-3}$ M) | 6350 ± 520 | 22.57 ± 2.13 | 2.17 ± 0.20 | 1.05 ± 0.10 |
| Amphetamine ($10^{-2}$ M) | 4440 ± 330† | 20.61 ± 2.24 | 2.28 ± 0.38 | 1.93 ± 0.14† |
| Amphetamine (10 mg/kg i.p.) | 3930 ± 410† | 23.10 ± 2.89 | 1.95 ± 0.24 | 3.49 ± 0.26† |

* Cortical tissue slices (0.26 mm thickness) were incubated in a Krebs-Ringer-Bicarbonate buffer in the presence of 3 μCi $^{14}C$-choline (spec. act. 54 mCi/m-mole) for 2 hr. *d*-Amphetamine was added to the incubation mixture or animals were pretreated with the drug 0.5 hr prior to sacrifice. After incubation, the levels of $^{14}C$-phosphatidylcholine, $^{14}C$-choline (Ch), $^{14}C$-phosphorylcholine (PCh) and $^{14}C$-cytidine diphosphorylcholine (CDPCh) were determined. $^{14}C$-phosphatidylcholine data are expressed as dpm/ mole of lipid P. The levels of $^{14}C$-Ch, $^{14}C$-PCh and $^{14}C$-CDPCh are given as dpm/g tissue wet weight. N = 5 to 6 determinations per group.

† Significantly different from control, $p < 0.05$.

## TABLE 20

### Effect of *d*-Amphetamine on the Incorporation of $^{14}$C-Choline Into Phosphatidylcholine in a Cortical Brain Homogenate *

| Group | $^{14}$C-phosphatidylcholine (dpm/μmole lipid P) |
|---|---|
| Control | 4160 |
| Amphetamine ($10^{-2}$ M) | 3940 |
| Amphetamine ($10^{-3}$ M) | 4060 |
| Amphetamine ($10^{-4}$ M) | 4250 |
| Amphetamine (10 mg/kg i.p.) | 3980 |

* A 10 percent cortical homogenate was prepared in 0.32 M sucrose and centrifuged at 1000 g for 10 min. One ml of the supernatant was suspended in 9 ml KRB buffer and 1 μCi $^{14}$C-choline was added. The incubation was performed for 2 hr in the presence of added *d*-amphetamine. In some experiments animals were pretreated with 10 mg/kg of *d*-amphetamine 0.5 hr prior to sacrifice. The amount of $^{14}$C-phosphatidylcholine formed was determined and the data are expressed as mean dpm/μmole of lipid P. N = 3 experiments per group.

base-exchange reaction which has an alkaline pH optimum. The data in Table 21 demonstrate that *d*-amphetamine in concentrations from $10^{-2}$ to $10^{-4}$ did not alter the rate of base-exchange. The experimental conditions used tend to minimize the cytidine dependent pathway of biosynthesis.

## 9.9 EFFECT OF *d*-AMPHETAMINE ON PHOSPHOLIPASE C ACTIVITY.

A portion of the cortical post 1000 g supernatant from animals pretreated 1 day with $^{14}C$-choline was incubated in KRB buffer in the presence of 20 μg/ml of phospholipase C. This concentration of enzyme produced a 75 percent reduction in $^{14}C$-phosphatidylcholine levels (Table 22). *d*-Amphetamine in concentrations from $10^{-2}$ to $10^{-4}$ M and pretreatment with 10 mg/kg 0.5 hr prior to sacrifice had no effect on phospholipase C activity.

## 9.10 DISCUSSION.

In the present experiments the effect of various treatments with *d*-amphetamine on the synthesis, turnover and metabolism of $^{14}C$-choline labeled phosphatidylcholine was investigated in discrete regions of the rat brain. The data demonstrate that (1) chronic *d*-amphetamine treatment inhibits $^{14}C$-phosphatidylcholine turnover in most brain regions and subcellular fractions, (2) acute *d*-amphetamine treatment inhibits the incorporation of $^{14}C$-choline into phosphatidylcholine in the cortex and cerebellum *in vivo*, (3) *d*-amphetamine inhibits the incorporation of $^{14}C$-choline into phosphatidylcholine in cortical slices and *d*-amphetamine does not appear to alter the metabolism of phosphatidylcholine. Possible mechanisms underlying these effects of *d*-amphetamine will be examined in some detail.

## TABLE 21

### Effect of *d*-Amphetamine on the Incorporation of $^{14}$C-Choline Into Phosphatidylcholine via Base-Exchange *

| Group | $^{14}$C-phosphatidylcholine (dpm/μmole lipid P) |
|---|---|
| Control | 1200 |
| Amphetamine ($10^{-2}$ M) | 1110 |
| Amphetamine ($10^{-3}$ M) | 1350 |

* A 10 percent cortical homogenate was prepared in 0.32 M sucrose and centrifuged at 1000 g for 10 min. A portion of the supernatant was suspended in 50 mM Tris buffer pH 9, containing 25 mM $CaCl_2$ and 1 μCi $^{14}$C-choline (spec. act. 54 mCi/m-mole). The incubation was performed for 2 hr in the presence of added *d*-amphetamine. The amount of $^{14}$C-phosphatidylcholine formed was determined and the data are expressed as dpm/μmole of lipid P. N = 3 experiments.

## TABLE 22

### Effect of *d*-Amphetamine on Phospholipase C Activity *

| Group | | $^{14}$C-phosphatidylcholine (cpm/μmole lipid P) |
|---|---|---|
| Control | | 2630 |
| Phospholipase C | (20 μg/ml) | 740 |
| | (50 μg/ml) | 570 |
| Phospholipase C<br>*d*-amphetamine | (30 μg/ml) +<br>($10^{-3}$ M) | 800 |
| | ($10^{-2}$ M) | 765 |
| | (10 mg/kg i.p.) | 690 |

* Animals were given 5 μCi $^{14}$C-choline i.cist. One day later the animals were sacrificed, a 10 percent cortical homogenate was made, and the homogenate was centrifuged 10 min at 1000 g. One ml of the supernatant was incubated with 9 ml KRB buffer and various additions of phospholipase C (*Cl. welchii*) and *d*-amphetamine. In some experiments animals were treated with 10 mg/kg of *d*-amphetamine 30 min prior to sacrifice. The incubation was terminated after 30 min by adding 2 ml of 50 percent TCA. After suitable washing procedures, the level of $^{14}$C-phosphatidylcholine in the TCA precipitate was determined. N = three experiments per group.

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The turnover of brain phospholipids is a complex process involving, at least, synthesis, metabolism, substrate availability, utilization and the metabolic activity of the structure(s) which contains the phospholipid. The experimental design of the present study is such that only the turnover of those pools of phosphatidylcholine with a $T_{\frac{1}{2}}$ value in the range of 4-28 days would be accurately measured. Burton (1969) has demonstrated that phospholipids labeled in the fatty acid moieties will show varying turnover rates depending on the structure in which they are contained. Phospholipids associated with the myelin sheath exhibited $T_{\frac{1}{2}}$ values in the range of 50-100 days, while those associated with cellular membranes exhibited turnover rates in the range of 7-38 days. Extrapolating this information to our study, it would appear that a single pulse label of $^{14}$C-choline primarily labels cellular membranes. The observation that little $^{14}$C-sphingomyelin could be detected confirms the relatively slow turnover and, consequently, low incorporative ability of myelin in the adult rat.

The present finding that chronic *d*-amphetamine treatment inhibited $^{14}$C-choline labeled phosphatidylcholine turnover in most brain regions and in the mitochondrial and microsomal fractions suggests a common mechanism of inhibition. The possibility that *d*-amphetamine could directly inhibit the incorporation of $^{14}$C-choline into phosphatidylcholine was investigated. Data on the effect of acute *d*-amphetamine treatment on the incorporation of $^{14}$C-choline into phosphatidylcholine *in vivo* show that *d*-amphetamine significantly inhibited incorporation, but only in the cortex and cerebellum. Some brain regions such as the hypothalamus and diencephalon showed a slightly increased incorporation. Interestingly, the large decrease in $^{14}$C-choline incorporation into phosphatidylcholine in the cerebellum after acute *d*-amphetamine treatment was not complemented by a large decrease in turnover in this brain region. These data suggest either that tolerance develops to the effects of *d*-amphetamine in the cerebellum or the drug affects phosphatidylcholine metabolism in this brain region or that the inhibition is an artifact of the experimental procedures employed. The magnitude of the *d*-amphetamine-induced decrease on the initial synthesis of $^{14}$C-phosphatidylcholine in the cortex is similar to the decrease in $^{14}$C-phosphatidylcholine turnover and suggests that the decrease in turnover may result from an accumulated inhibitory effect on synthesis. However, different mechanisms are probably involved in the other brain regions. For example, the decreased turnover of $^{14}$C-phosphatidylcholine in non-cortical regions may result secondarily from the fact that *d*-amphetamine inhibits brain protein synthesis (see Chapter 11).

The mechanism by which acute *d*-amphetamine treatment inhibits $^{14}$C-choline incorporation into phosphatidylcholine in the cortex was further investigated. The addition of high concentrations of *d*-amphetamine ($10^{-2}$ M) or pretreatment with 10 mg/kg of drug inhibited the synthesis of $^{14}$C-phosphatidylcholine in cortical slices. Examination of the formation of $^{14}$C-choline metabolites in these experiments indicated that *d*-amphetamine caused $^{14}$C-cytidine diphosphorylcholine to accumulate in the tissue, suggesting that the drug interferes with the formation of phosphatidylcholine at the level of cytidine diphosphorylcholine diglyceride transferase. However, *d*-amphetamine was not found to alter the formation of $^{14}$C-phosphatidylcholine in a cortical homogenate. These data indicate that the mechanism by which *d*-amphetamine alters phosphatidylcholine formation at the level of the cytidine diphosphorylcholine diglyceride transferase or perhaps other biosynthetic steps is dependent on an intact tissue preparation.

The mechanism by which *d*-amphetamine inhibits the turnover of $^{14}C$-phosphatidylcholine in the cortex and other brain regions may involve not only a drug effect on synthesis but also an effect on metabolism. The non-enzymatic base-exchange reaction may be considered both a metabolic and a biosynthetic pathway. Base-exchange has been shown to be an important mechanism for the turnover of the base components of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine in brain homogenate and nerve ending preparation (Porcellati and diJeso, 1971; Abdel-Latif and Smith, 1972). In the present study, *d*-amphetamine was not found to alter the incorporation of $^{14}C$-choline into phosphatidylcholine under experimental conditions which maximize the base-exchange reaction.

In addition to base-exchange, choline can be removed from phosphatidylcholine by the activity of phospholipase C. To examine the effect of *d*-amphetamine on this enzyme, animals were pretreated with $^{14}C$-choline and the effect of a purified phospholipase C preparation on the content of cortical $^{14}C$-phosphatidylcholine was measured. Under these conditions, *d*-amphetamine was not found to alter enzyme activity. However, since the drug effect on the brain enzyme was not measured, the possibility cannot be excluded that *d*-amphetamine has some action on brain phospholipase C activity.

The possible physiological significance of the *d*-amphetamine induced decrease in phosphatidylcholine turnover has been discussed in the introduction. Briefly, the present study supports our original hypothesis that one mechanism by which *d*-amphetamine can produce a "permanent" increase in NE release is by decreasing the turnover of the phosphorylcholine moiety of phosphatidylcholine. Further testing of the hypothesis might include examining the effects of other chronic drug treatments on phosphatidylcholine turnover and NE release. For the purposes of this study, the effects of *d*-amphetamine on phosphatidylcholine turnover have been expanded to include measurement of the drug effects on synaptic plasma membranes (SPM) prepared from discrete populations of nerve ending particles. These data are reported in the next chapter. The exact subcellular site where the effect of *d*-amphetamine on turnover should influence release is the SPM.

# CHAPTER 10

# EFFECT OF CHRONIC AMPHETAMINE INTOXICATION ON PHOSPHATIDYLCHOLINE TURNOVER IN SYNAPTIC PLASMA MEMBRANES

## 10.1 INTRODUCTION.

In the previous chapter it was observed that chronic amphetamine intoxication inhibited the turnover of $^{14}$C-choline labeled phosphatidylcholine in the crude mitochondrial fraction of several brain regions. This inhibitory effect may be of some importance in explaining the persistent increase in the release of newly synthesized NE (and DA) following withdrawal of amphetamine (see Chapters 7, 8 and 9). However, changes in turnover occurring in the mitochondrial fraction can roughly be used as an index of changes occurring in nerve endings and more particularly the limiting synaptic plasma membrane (SPM). The concept developed in Chapter 8 suggests that when the phosphorylcholine moiety of phosphatidylcholine in the external surface of the SPM becomes dysfunctional, the spontaneous release of NE will increase. Thus, the amphetamine-induced decrease in phosphatidylcholine turnover must occur at least in the SPM in order for the correlation between release and turnover to exist.

In Chapters 2 and 3 techniques were described which permit the separation of different classes of nerve ending particles (NEP) on the basis of their relative ability to concentrate labeled GABA and NE. To be sure, the population of NEP which concentrate NE are not purely noradrenergic in character; other kinds of NEP will contaminate the NE-NEP rich fraction. However, the noradrenergic rich NEP may be of sufficient purity to detect biochemical changes which are peculiar to noradrenergic NEP. In this chapter the effect(s) of chronic amphetamine intoxication on the turnover of $^{32}P$-$^{3}H$-choline labeled phosphatidylcholine in discrete populations of SPM derived from light (GABA) and heavy (NE) NEP are described.

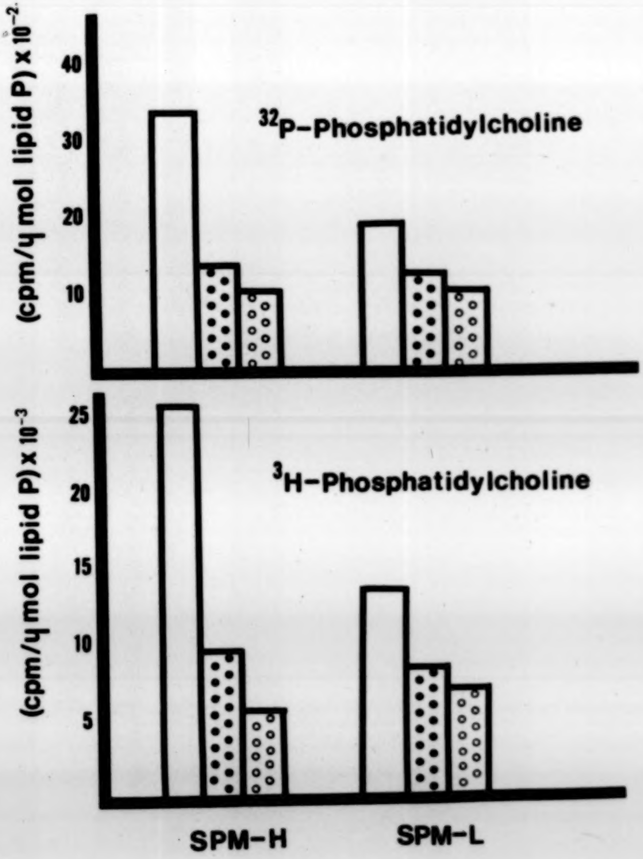
## 10.2 EXPERIMENTAL - CHEMICAL.

Male Sprague-Dawley rats (Horton Laboratories, Oakland, Cal.) weighing 150 to 200 g were implanted with a permanent cannulae guide into either the left or right ventricle under light pentobarbital anesthesia. Three to 5 days later the animals were administered 100 μCi of $^{32}P$ and 20 μCi of $^{3}H$-choline (13.0 Ci/mM) dissolved in 20 μl of KRB buffer. One week later the animals were put on a staircase injection schedule of *d*-amphetamine (high dose schedule) similar to that described in Chapter 9 except that each dose level was maintained for 1 week instead of 5 days. Animals were sacrificed at 1 and 4 weeks and SPM were prepared from the subcortex as described in Section 3.4. Phosphatidylcholine was extracted from the SPM and its specific activity determined as described in Section 3.5.

## 10.3 EFFECT OF CHRONIC AMPHETAMINE INTOXICATION ON PHOSPHATIDYLCHOLINE TURNOVER IN LIGHT (GABA) AND HEAVY (NE) SPM.

The data in Fig. 45 illustrate that 1 week after administration of $^{32}P$ and $^{3}H$-choline, the $^{3}H$ and $^{32}P$ specific activity of phosphatidylcholine was significantly higher, 60 percent ($p < 0.05$), in the SPM-H as compared to the SPM-L. Although the time course of these experiments was limited to approximately one $t_{\frac{1}{2}}$ period, it appears that the SPM-L have a faster turnover rate than the SPM-H. Chronic amphetamine adminis-

Fig. 45. Effect of chronic amphetamine on the disappearance of $^{32}$P-$^{3}$H-choline labeled phosphatidylcholine from two populations of SPM. Rats were administered 100 μCi $^{32}$P and 20 μCi $^{3}$H-choline i.vent. One week later the animals were put on the chronic injection schedule of amphetamine described in Methods. After 3 weeks of injections, the animals were sacrificed, SPM-L and SPM-H were prepared from the subcortex and phosphatidylcholine was isolated. Data are the mean of two experiments. The values obtained for the two experiments differed less than 5 percent. □ = 1 week control, ▣ = 4 week control; ⊡ = 4 week amphetamine treated.


(cpm/μmol lipid P) x 10⁻²
40
30
20
10
³²P–Phosphatidylcholine
(cpm/μmol lipid P) x 10⁻³
25
20
15
10
5
³H–Phosphatidylcholine
SPM–H
SPM–L

tration only slightly enhanced the rate of disappearance of $^3$H and $^{32}$P labeled phosphatidylcholine isolated from the SPM-L. However, the chronic drug treatment markedly enhanced the disappearance of $^3$H-phosphatidylcholine isolated from the SMP-H. An effect of similar magnitude was not observed for SPM-H $^{32}$P-phosphatidylcholine.

## 10.4 DISCUSSION.

In the previous chapter it was shown that chronic amphetamine treatment produced a fairly marked inhibition of $^{14}$C-phosphatidylcholine turnover in the crude mitochondrial and microsomal fractions derived from 6 brain regions. The same amphetamine treatment had no effect on the disappearance of $^{32}$P and $^3$H-choline labeled phosphatidylcholine isolated from the SPM-L (GABA). The fact that chronic amphetamine treatment enhanced the disappearance of $^3$H-(choline)-phosphatidylcholine in SPM derived from NEP which concentrate NE negates the hypothesis stated in Chapter 9 "that one possible mechanism by which chronic amphetamine increases the spontaneous release of newly synthesized NE is to decrease the turnover of the phosphorylcholine moiety of phosphatidylcholine". Thus, it must be concluded that the persistent increase in release results from a yet unknown physiological-biochemical adaptation. The importance of the decrease in turnover in the mitochondrial and microsomal fractions (Chapter 9) should not be discounted as having no chemical or behavioral significance. It would not be unreasonable, for example, to assume that a decrease in phosphatidylcholine turnover will have a marked effect on the activity of those enzymes for which phosphatidylcholine exerts a regulatory role (e.g., see Bruni *et al.*, 1971). At this time, though, I have not measured nor wish to speculate what these possible changes may be.

## 10.5 GENERAL COMMENT.

The possibility must be considered that the reason for the different results on phosphatidylcholine turnover obtained in Chapters 9 and 10 lies in the fact that the specific activity of the labeled choline was changed. Ideally, the specific activity of the precursor in pulse labeling experiments should be maintained as low as is possible in order to decrease the possibility that a high substrate concentration will influence the velocity of the reaction and to obtain maximum mixing of the endogenous and exogenous substrate. Thus, $^{3}$H-choline would be preferred over $^{14}$C-choline with its lower specific activity. $^{14}$C-Choline was used for the experiments described in Chapter 9 since the $^{14}$C-choline was mixed with $^{3}$H-leucine in order to simultaneously measure protein and phosphatidylcholine turnover. The data on ($^{3}$H-leu)-protein turnover is presented in the next chapter. Although in turnover studies the effect of using $^{14}$C-choline will not directly affect the rate of turnover as it would affect the rate of synthesis, the possibility is strong that $^{14}$C-choline could label a different store of phosphatidylcholine. $^{14}$C-Choline will increase the total brain choline pool and thus maintain the level of labeled choline in the brain for a longer time than with $^{3}$H-choline. This could have the effect to label more of the slowly turnover over stores of phosphatidylcholine. Furthermore, using $^{14}$C-choline rather than $^{3}$H-choline will decrease the effectiveness and uniformity of the mixing process. Thus, in future studies I would advise using the highest specific activity precursor available for any pulse labeling experiment in order to eliminate the problems outlined above that could occur when $^{14}$C-choline is used to measure phosphatidylcholine turnover.

# CHAPTER 11

# EFFECT OF *d*-AMPHETAMINE ON BRAIN PROTEIN SYNTHESIS AND TURNOVER

## 11.1 INTRODUCTION.

In attempting to explain his data on amphetamine-induced abnormalities in sleeping behavior, Oswald (1970) suggested that amphetamine may influence brain protein synthesis and in this way produce "permanent" alterations in brain physiology-chemistry. A similar hypothesis could be presented for almost any of the "permanent" behavioral effects that result from chronic amphetamine intoxication, e.g., psychosis (see Kalant, 1966). As a beginning to investigate this hypothesis, it would seem necessary to establish in at least a general manner what effect amphetamine has on brain protein synthesis and turnover, if any. The studies described in this chapter were designed to determine the status of the brain protein synthetic machinery after acute and chronic *d*-amphetamine treatment. Two separate studies are described. In the first study the effect of acute amphetamine intoxication on cell-free protein synthesis was examined. The second study was similar to that reported in Chapter 9 except that $^3$H-leucine was used to determine the effect of amphetamine on protein synthesis and turnover in discrete regions of the rat brain.

## 11.2 EXPERIMENTAL *In vitro* PROTEIN SYNTHESIS.

Male albino mice (Horton Laboratories) received single injections of *d*-amphetamine sulfate i.p. in doses ranging from 5 to 100 mg/kg, while the control animals received equivalent volumes of 0.9% saline.

In order to avoid an excessive mortality rate which usually occurs during aggregation, all animals including the controls were isolated into separate cages following drug pretreatment. Mice were sacrificed by decapitation at intervals ranging from 1 to 24 hr. Brain tissue was removed and homogenized in 4 volumes of medium I. Microsomes and soluble fraction were prepared and protein synthesis was assayed as described by Zomzely *et al.* (1968) (see Chapter 3).

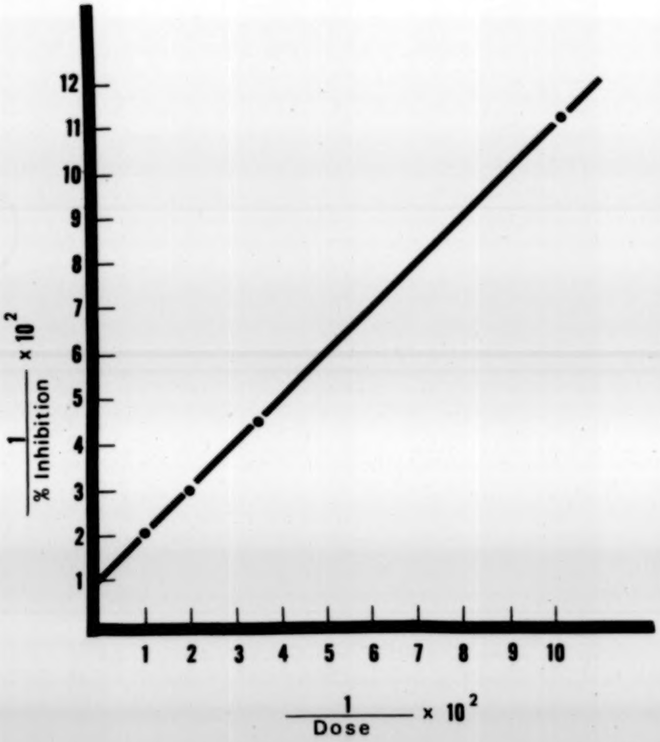
## 11.3 EXPERIMENTAL - *In vivo* PROTEIN SYNTHESIS AND TURNOVER.

The experimental design used to measure $^3$H-protein synthesis and turnover is exactly the same as that described for measuring $^{14}$C-phosphatidylcholine synthesis and turnover in Chapter 9 except that the rats were administered 20 μCi of $^3$H-leucine (specific activity, 30 Ci/mmol) intracisternally. The schedule of *d*-amphetamine injections in both the acute experiments and the turnover studies was also the same as described in Chapter 9. After dissection of the brains and preparation of the subcellular fractions, $^3$H-protein was isolated on Whatman GFA filters as described in Chapter 3. The counting efficiency of the $^3$H-protein was estimated to be 10 percent.

## 11.4 EFFECT OF ACUTE AMPHETAMINE INTOXICATION ON *In vitro* PROTEIN SYNTHESIS.

Like the central nervous system agents reserpine (Stolman and Loh, 1971), ethanol (Kuriyama *et al.*, 1971) and morphine (Clouet and Ratner, 1967), toxic doses of *d*-amphetamine inhibit the incorporation of uniformly radiolabeled leucine into brain microsomal protein. As shown in Fig. 46, there was a 17, 31 and 45 percent inhibition for the 30, 50 and 100 mg/kg i.p. doses, respectively, 1 hr after drug pretreatment. Doses

Fig. 46. Effect of increasing doses of *d*-amphetamine on *in vitro* brain microsomal protein synthesis. Male Swiss-Webster mice were treated with various doses (10-100 mg/kg i.p.) of *d*-amphetamine. One hr later the animals were sacrificed, brains were removed and microsomes (Mc) and soluble fraction (SF) were prepared and incubated in buffer containing 1 μCi of [$^{14}$C]leucine. After a 30 min incubation the amount of radiolabeled protein formed was determined. Data are presented as a 1/% inhibition from control vs. 1/dose of *d*-amphetamine in mg/kg. N = 5-6 experiments per *d*-amphetamine dose.


12
11
10
9
8
7
6
5
4
3
2
1
1/% Inhibition x 10^2
1
2
3
4
5
6
7
8
9
10
1/Dose x 10^2

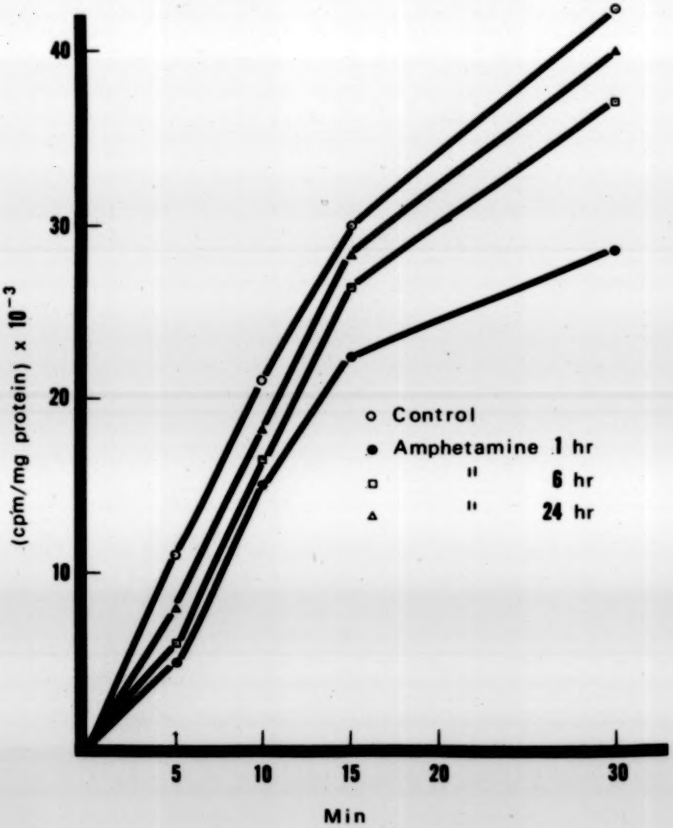
of *d*-amphetamine less than 30 mg/kg did not alter the rate of incorporation of leucine into protein as compared to the controls. Furthermore, the inhibition produced by a 30 mg/kg dose was reversible. As illustrated in Fig. 47, optimal inhibitory effects evoked 1 hr after injection gradually returned to normal rates of synthesis between 6 and 24 hr after injection.

The behavior of the animals receiving 30 mg/kg of *d*-amphetamine or larger doses at the time of maximal inhibition of protein synthesis (1 hr after drug treatment) was that of highly repetitive licking, gnawing and biting movements, arrhythmic shaking of the whole body, profuse sweating and extreme hyperthermia. The rectal temperature of the animals given a dose of 50 mg/kg increased from 37.2° C to 42.0° C. The dose of 100 mg/kg was lethal to more than 90 percent of the animals tested within 2 to 3 hr of drug treatment.

The data presented in Table 23 illustrate that *d*-amphetamine inhibits protein synthesis when added to a control incubation system. Ten millimolar amphetamine caused a 23 percent inhibition of protein synthesis.

In order to delineate whether the acute inhibitory effect produced by *d*-amphetamine *in vivo* could be attributed to its action on the microsomal or soluble fraction, cross incubation experiments were conducted. The data presented in Fig. 48 show no appreciable change in the rate of protein synthesis in microsomes prepared from amphetamine-treated mice in combination with the control soluble fraction. On the other hand, a significant reduction in the rate of protein synthesis occurred when microsomes from control mice were incubated with soluble fraction from amphetamine-treated mice.

Fig. 47. Time course of the effects of *d*-amphetamine on *in vitro* brain microsomal protein synthesis. Male Swiss-Webster mice were administered saline or 30 mg/kg i.p. *d*-amphetamine. At 1, 6 and 24 hr after drug injection, the animals were sacrificed and microsomal protein synthesis was determined. The amount of radiolabeled protein formed was determined at 5, 10, 15 and 30 min after incubation. N = 5-6 experiments per time point.


(cpm/mg protein) x $10^{-3}$
40
30
20
10
5
10
15
20
30
Min
○ Control
● Amphetamine 1 hr
□ " 6 hr
△ " 24 hr

XUM

### TABLE 23

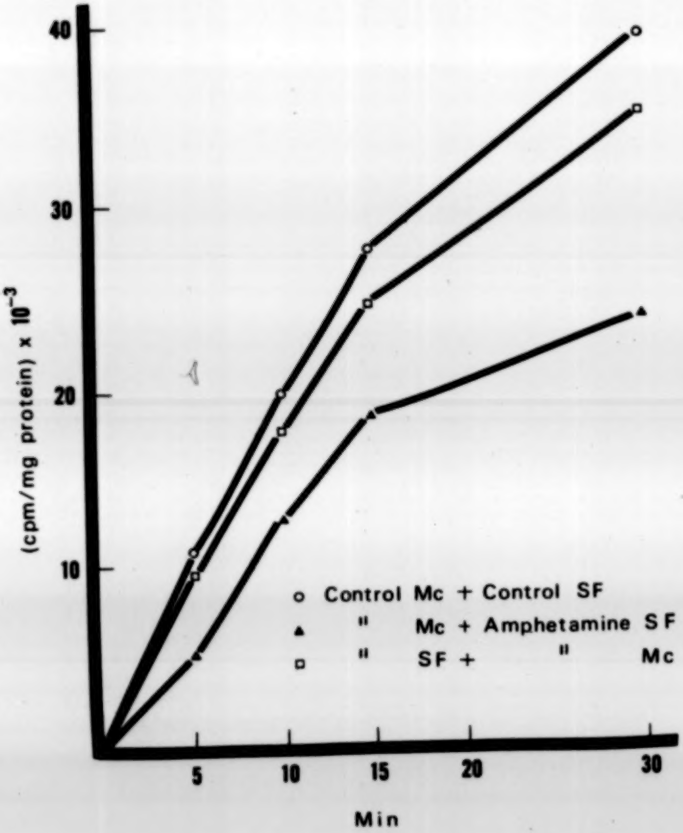
### Effect of Amphetamine on the *In vitro* Incorporation of L-[U-$^{14}$C]Leucine into Brain Microsomal Protein[a]

| Amphetamine Concentration (mM) | Mean ± S.E. (cpm/mg protein/30 min) x $10^{-3}$ |
|---|---|
| 10 | 28.8 ± 1.40 |
| 5 | 30.8 ± 1.23 |
| 1 | 31.1 ± 1.17 |
| 0.5 | 35.0 ± 1.32 |
| 0.1 | 36.3 ± 1.35 |
| None | 37.5 ± 1.26 |

---

[a] Microsomes and soluble fraction were prepared from the whole mouse brain, and portions of each were incubated in the presence of [$^{14}$C] leucine. The effect of the addition of various concentrations of *d*-amphetamine to the incubation mixture on the incorporation of [$^{14}$C]leucine into microsomal protein was measured. N = 5-6 experiments per amphetamine concentration.

Fig. 48. Effect of cross-incubation on the *d*-amphetamine-induced inhibition of *in vitro* brain microsomal protein synthesis. Male Swiss-Webster mice were injected with saline or 30 mg/kg i.p. *d*-amphetamine. One hr later Mc and SF were prepared and cross-incubation experiments were performed. Data are presented as cpm/mg protein vs. min after beginning of incubation. N = 5-6 experiments per time point.



(cpm/mg protein) x $10^{-3}$
40
30
20
10
5
10
15
20
30
Min
○ Control Mc + Control SF
▲ " Mc + Amphetamine SF
□ " SF + " Mc

## 11.5 DISCUSSION OF THE EFFECTS OF *d*-AMPHETAMINE ON *In vitro* PROTEIN SYNTHESIS.

Previous studies have indicated that the stereotyped behaviors and shaking movements are not blocked by pretreatment with α-methyltyrosine and/or reserpine (Hitzemann, 1970; Stolk and Rech, 1970), suggesting that the behavioral effects caused by large doses of *d*-amphetamine are apparently related to a direct drug effect rather than an indirect action of the drug on the catecholamines. In the present study the linear relationship between the dose of amphetamine and the incidence of protein synthesis inhibition suggests that the inhibitory effect was associated with the brain drug level. Furthermore, the time course of inhibition (see Fig. 47) parallels the time course of the disappearance of *d*-amphetamine from mice administered a large drug dose (Hitzemann *et al.*, 1970). However, if the inhibition of microsomal protein synthesis was solely related to the presence of the drug in the brain, it should be possible to simulate the effects of *in vivo* drug administration with the *in vitro* additions of drug to the incubation mixture. Such was not the case. The concentrations of *d*-amphetamine needed to inhibit protein synthesis *in vitro* are at least one to two orders of magnitude greater than those drug levels which produce toxicity and adverse behavioral effects (Hitzemann, 1970). These data suggest that the inhibitory response produced by *d*-amphetamine administered *in vivo* may be related to the marked stimulation of the central nervous system caused by amphetamine (Sellinger and Azcurra, 1970). In addition, toxic doses of amphetamine have been shown to produce convulsions, marked edema of the brain and gross dilation of the lateral and third ventricles (Kalant, 1966).

The data obtained from the cross-incubation experiments suggest that the *d*-amphetamine-induced inhibition of protein synthesis cannot be attributed to a decrease of transferase activity and/or an augmented disaggregation of polysomes attached to the endoplasmic reticulum. The most likely mechanism for the inhibitory effect involving the soluble fraction is a drug-induced suppression of aminoacylation of soluble-RNA rather than alteration of ATP synthesis or pool size, since the inhibitory effect still ensues in the presence of optimal ATP levels *in vitro*. One possible additional mechanism of inhibition is that amphetamine has modified the levels of free amino acids in the soluble fractions. Reports from several laboratories have shown that specific amino acid substrates have stimulatory or inhibitory effects on brain protein synthesis in a cell free system (Hanking and Roberts, 1965; Orrego and Lipman, 1967; Roberts and Morelos, 1965). Also, a specific increase in leucine would decrease the $^{14}$C-leucine specific activity and result in an apparent decrease in protein synthesis.

The results obtained from these studies clearly demonstrate that *d*-amphetamine, albeit high doses of the drug, can alter brain protein synthesis in an artificial system. The question then arose as to whether or not *d*-amphetamine has a similar effect on protein synthesis *in vivo*. Two studies were conducted in this regard. In the first study the effect of acute doses of *d*-amphetamine on the initial incorporation of $^{3}$H-leucine into protein was examined. In the second study the effect of repeated doses of *d*-amphetamine on the turnover of $^{3}$H-leucine labeled protein was examined.

## 11.6 EFFECT OF ACUTE *d*-AMPHETAMINE TREATMENT ON THE *In vivo* SYNTHESIS OF ($^3$H-LEU)-PROTEIN.

Rats were administered either 1 or 10 mg/kg of *d*-amphetamine i.p. 0.5 hr prior to the administration of 20 μCi of $^3$H-leucine. Thirty min after leucine injection the animals were sacrificed and the levels of unincorporated $^3$H and ($^3$H-leu)-protein were determined in discrete brain regions. The results of these studies are given in Table 24. Neither dose of *d*-amphetamine significantly affected the levels of unincorporated $^3$H in any brain region. This lack of effect is in marked contrast to the data obtained with other psychoactive drugs, e.g., morphine (Clouet and Neidle, 1970; Hitzemann and Loh, 1974) and pentobarbital (unpublished observation).

Preliminary experiments indicated that the incorporation of $^3$H-leucine into protein increases linearly for at least 45 min after the injection of $^3$H-leucine. For this reason, 30 min was chosen as a convenient time to examine the effects of *d*-amphetamine on ($^3$H-leu)-protein accumulation. One mg/kg of *d*-amphetamine significantly increased ($^3$H-leu)-protein accumulation in the cortex, cerebellum and hypothalamus. Ten mg/kg of *d*-amphetamine significantly inhibited ($^3$H-leu)-protein accumulation in the cortex, cerebellum, caudate nucleus and diencephalon.

## 11.7 EFFECT OF *d*-AMPHETAMINE ON THE TURNOVER OF ($^3$H-LEU)-PROTEIN.

Various reports (see review, Lajtha and Marks, 1969) have demonstrated that the bulk of brain proteins have half lives ($t_{1/2}$) ranging from 7 to 21 days with the average being about 14 days. The values obtained depend on a variety of factors including the isotope used, route of isotope administration, brain region and subcellular fraction investigated and the length of time used to calculate the $t_{1/2}$ values.

TABLE 24

Effect of Acute *d*-Amphetamine Treatment on the Synthesis of ($^3$H-Leu)-Protein [a]

| Brain Region | Dose of *d*-Amphetamine (mg/kg) | TCA Soluble $^3$H (dpm/g ± S.E.) x $10^{-4}$ | $^3$H-Protein (dpm/mg protein) x $10^{-1}$ |
|---|---|---|---|
| Cortex | 0 | 16.9 ± 3.5 | 11.2 ± 1.9 |
| | 1 | 15.3 ± 2.2 | 19.6 ± 0.5* |
| | 10 | 14.6 ± 3.8 | 5.4 ± 1.1*,** |
| Cerebellum | 0 | 88.9 ± 6.2 | 108.7 ± 18.4 |
| | 1 | 89.2 ± 12.6 | 187.3 ± 11.8* |
| | 10 | 84.3 ± 9.8 | 68.2 ± 5.4*,** |
| Hypothalamus | 0 | 6.4 ± 0.9 | 47.8 ± 10.5 |
| | 1 | 6.7 ± 1.0 | 87.9 ± 4.6* |
| | 10 | 5.6 ± 1.2 | 47.5 ± 2.7** |
| Caudate Nucleus | 0 | 13.5 ± 2.1 | 11.2 ± 0.5 |
| | 1 | 12.8 ± 1.7 | 13.1 ± 1.4 |
| | 10 | 11.4 ± 1.6 | 3.8 ± 1.0*,** |
| Brain Stem | 0 | 103.2 ± 10.4 | 298.0 ± 51.4 |
| | 1 | 134.8 ± 14.4 | 312.5 ± 21.7 |
| | 10 | 121.2 ± 12.9 | 233.0 ± 20.7** |
| Diencephalon | 0 | 36.9 ± 3.0 | 72.9 ± 5.1 |
| | 1 | 41.2 ± 14.6 | 97.5 ± 17.1 |
| | 10 | 40.9 ± 8.2 | 47.7 ± 12.4*,** |

[a] Rats were administered *d*-amphetamine i.p. 0.5 hr prior to the injection of 20 μCi of $^3$H-leucine (specific activity 20 Ci/mmol) i.cist. 0.5 hr later the animals were sacrificed and the levels of TCA soluble $^3$H and $^3$H-protein were determined in discrete brain regions. N = 6 animals per drug dose. * - Significantly different than control, $p < 0.05$. ** - Significantly different than 1 mg/kg of *d*-amphetamine, $p < 0.05$.

It is probably necessary to have the time course of the experiment run at least three $t_{\frac{1}{2}}$ periods in order to accurately assess a true $t_{\frac{1}{2}}$ value. The experimental period used in the present study was limited to only 15 days or approximately 1 $t_{\frac{1}{2}}$ period. As suggested by the foregoing argument, this period should be sufficiently long to compare turnover rates in normal and drug treated animals but cannot serve as an accurate determinate of the absolute turnover rates.

Realizing the limitations of the techniques used, it is interesting to note that the $t_{\frac{1}{2}}$ values for the mitochondrial, microsomal and soluble fractions are both similar and uniform in all brain regions except the brain stem (Fig. 49 to 51 and Table 25). In this brain region the $t_{\frac{1}{2}}$ values for the microsomal and soluble fractions are larger than in other areas indicating a slower turnover. The possibility that i.cist. injection affected turnover in this region must be considered. The turnover of soluble protein in the cerebellum ($t_{\frac{1}{2}}$ = 22 days) was also found to be slower than the rate generally observed ($t_{\frac{1}{2}}$ = 9 to 12 days). The effect of *d*-amphetamine administered in a staircase schedule on ($^3$H-leu)-protein turnover is summarized in Table 25. Chronic amphetamine treatment inhibited ($^3$H-leu)-protein turnover in the crude mitochondrial fraction of all brain regions examined. The inhibition of turnover was greatest in the hypothalamus, caudate nucleus and cerebellum. The turnover of microsomal and soluble protein was increased, decreased or showed no change after chronic amphetamine treatment depending on the brain region investigated. In the microsomal fraction the greatest decrease in turnover occurred in the hypothalamus and greatest increase in turnover occurred in the diencephalon. In the soluble fraction amphetamine markedly decreased turnover in the brain stem and diencephalon.

Fig. 49. Turnover of ($^3$H-leu)-protein in the crude mitochondrial fraction of the cortex, caudate nucleus, diencephalon, cerebellum, brain stem and hypothalamus from normal and chronic amphetamine treated animals. Animals were administered 20 µCi of $^3$H-leucine (spec. act. 25 Ci/mmol) i.cist. Beginning 5 days after label administration, animals were administered 1 mg/kg *d*-amphetamine i.p. twice daily. The dose of amphetamine was doubled 5 days later and doubled once again 10 days later. Control groups were sacrificed on days 5, 10, 15 and 20. Amphetamine treated groups were sacrificed 12 hr after their last injection on days 10, 15 and 20. Data in Fig. 49 are plotted as log dpm/mg protein ± S.E. vs. time. Each point represents the mean of 6 determinations. * - Significantly different than control on day 20 - $p < 0.05$. ●-● - control; ▲-▲ - amphetamine treated.

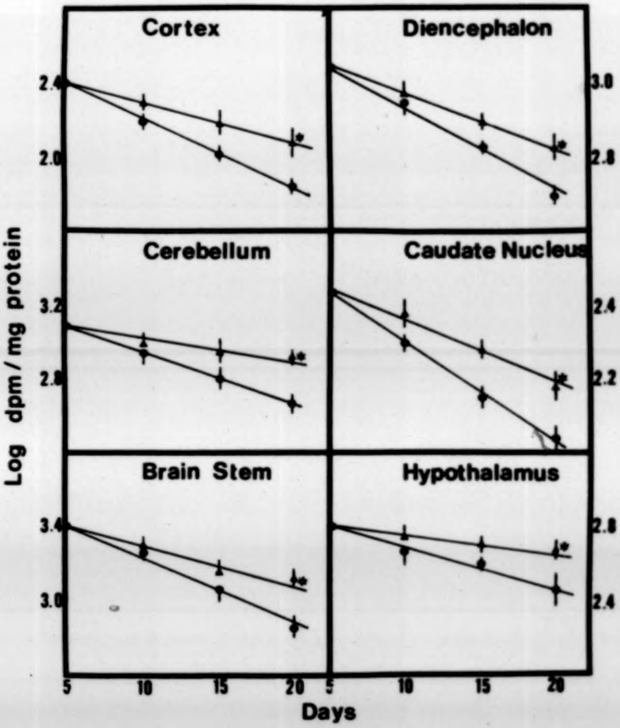

Cortex
Diencephalon
Cerebellum
Caudate Nucleus
Brain Stem
Hypothalamus
Log dpm / mg protein
2.4
2.0
3.0
2.8
3.2
2.8
2.4
2.2
3.4
3.0
2.8
2.4
*
5
10
15
20
Days

Fig. 50. Turnover of ($^3$H-leu)-protein in the microsomal fraction from discrete regions of the rat brain from normal and chronic amphetamine treated animals. Details are given in the legend to Fig. 49.

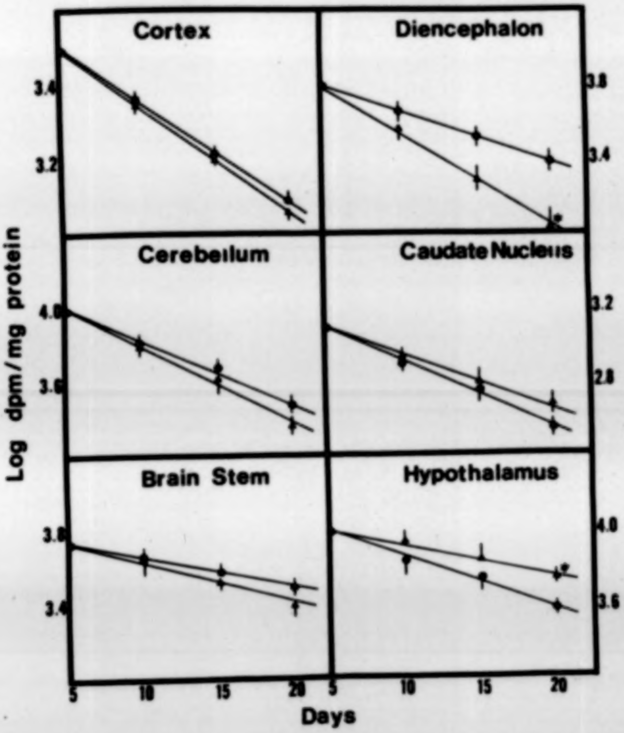
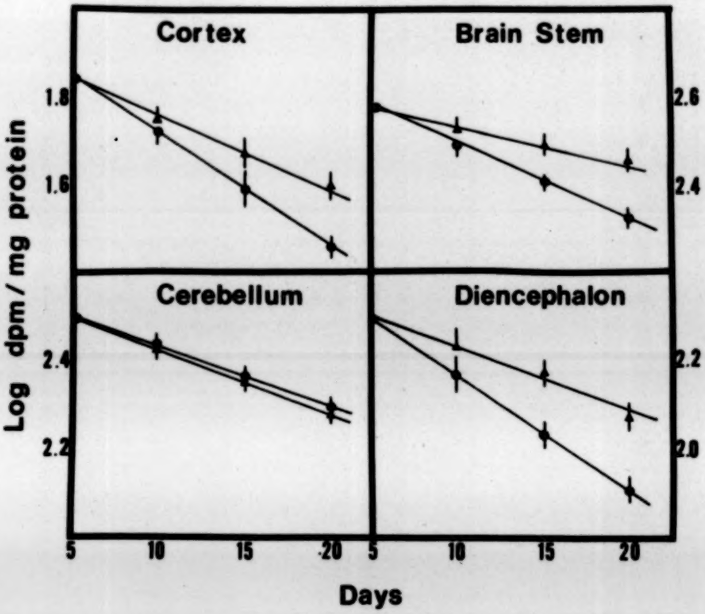

Cortex
Diencephalon
Cerebellum
Caudate Nucleus
Brain Stem
Hypothalamus
Log dpm/mg protein
Days
3.4
3.2
3.8
3.4
4.0
3.6
3.2
2.8
3.8
3.4
4.0
3.6
5
10
15
20
5
10
15
20
*
*

Fig. 51. Turnover of ($^3$H-leu)-protein in the soluble fraction from discrete regions of the rat brain from normal and chronic amphetamine treated animals. Details are given in the legend to Fig. 49.


Cortex
Brain Stem
Cerebellum
Diencephalon
Log dpm / mg protein
1.8
1.6
2.6
2.4
2.4
2.2
2.2
2.0
5
10
15
20
5
10
15
20
Days

## TABLE 25

### Effect of Chronic *d*-Amphetamine Treatment on the Turnover of ($^3$H-Leu)-Protein [a]

| | $t_{1/2}$ (days) | | | | | |
|---|---|---|---|---|---|---|
| | Mitochondrial | | Microsomes | | Soluble | |
| Brain Region | Control | Amphetamine | Control | Amphetamine | Control | Amphetamine |
| Cortex | 9 | 16* | 11 | 10 | 11 | 17* |
| Cerebellum | 11 | 22* | 12 | 10 | 22 | 20 |
| Brain Stem | 10 | 15* | 18 | 14 | 17 | 36* |
| Diencephalon | 10 | 14* | 12 | 6* | 11 | 20* |
| Caudate Nucleus | 9 | 18* | 9 | 10 | N.D. | N.D. |
| Hypothalamus | 9 | 24* | 12 | 20* | N.D. | N.D. |

[a] Rats were administered 20 µCi $^3$H-leucine i.cist. Twice daily injections of *d*-amphetamine were begun 5 days later and continued for 15 days. Injections were begun at 1 mg/kg i.p. and doubled every 5 days. Animals were sacrificed at various times after beginning amphetamine injection and the levels of ($^3$H-leu)-protein were determined in the crude mitochondrial, microsomal and soluble fractions prepared from the various brain regions. Half-life values ($t_{1/2}$) were determined by linear regression. The turnover of soluble protein in the hypothalamus and caudate nucleus was not determined (N.D.) due to the low levels of ($^3$H-leu)-protein obtained. * - The turnover rate was determined to be significantly different when the protein specific activity on day 20 for the amphetamine group was significantly different ($p < 0.05$) than control.

## 11.8 DISCUSSION OF THE EFFECTS OF *d*-AMPHETAMINE ON ($^3$H-LEU)-PROTEIN SYNTHESIS AND TURNOVER.

The hypothesis of Oswald (1970) that brain proteins are altered during chronic amphetamine treatment is supported by the present study. Furthermore, the fact that *d*-amphetamine primarily inhibits ($^3$H-leu)-protein turnover may serve as a possible explanation for the mechanism by which chronic amphetamine inhibits phosphatidylcholine turnover. Further considerations of the effect of *d*-amphetamine on brain protein turnover and synthesis can only be made in general terms since the drug effects were measured only on large groups rather than individual proteins.

Psychoactive drugs are known to produce regional effects on neurotransmitter synthesis and turnover. By analogy, it must be considered possible, if not probable, that a psychoactive drug such as amphetamine will also have regional effects on protein synthesis and turnover. The present experiments demonstrate that this is indeed the case. One mg/kg of *d*-amphetamine, a dose which produces mild psychomotor stimulation, increased the acute accumulation of ($^3$H-leu)-protein only in the cortex, cerebellum and hypothalamus. The other brain regions were not affected by this dose. Similarly, when the dose of amphetamine was increased to 10 mg/kg, inhibition of ($^3$H-leu)-protein accumulation was observed in most but not all brain regions.

The mechanism(s) underlying the dose-related increases or decreases in ($^3$H-leu)-protein accumulation are presently not clear. The possibility that amphetamine has altered the rate and not the maximal amount of ($^3$H-leu)-protein accumulation must be considered; extensive time course studies could elucidate this point. The decrease in ($^3$H-leu)-protein accumulation caused by 10 mg/kg and the decrease in ($^3$H-leu)-protein turnover observed primarily in the crude mitochondrial fraction may be related to those same factors that caused the inhibition of *in vitro* protein synthesis. However, it is important to remember that the *in vitro* system is artificially enriched in ATP, GTP and some ions. The situation *in vivo* which leads to the decrease in protein synthesis may be caused by a number of factors that are not encountered in the *in vitro* system, e.g., the distribution of amino acids, availability of ATP or GTP and so on.

The pulse labeling technique employed to estimate turnover rates of the brain proteins has been used by others with $^3$H-leucine as the substrate (Von Hungen *et al.*, 1968). The principle of this technique rests on the assumption that in the adult rat the rate of protein breakdown equals the rate of synthesis, i.e., a steady-state exists, and where there is little re-utilization of label the disappearance of the ($^3$H-leu)-protein may be used as an index of the rate of synthesis (or breakdown). $^3$H-Leucine is rapidly metabolized by a number of systems so that by 5 days after injection there is an insignificant amount of unincorporated $^3$H remaining in the brain (data not shown). Thus, re-utilization of label is likely to be of little consequence. However, the fact that the $^3$H-leucine is rapidly metabolized presents an additional problem of label entering into protein that is not $^3$H-leucine. Clouet (1970) has observed that 30 min after the injection of $^{14}$C-leucine, a time when the majority of the unincorporated label is $^{14}$C-glutamic acid, the only label in protein was $^{14}$C-leucine. These data would tend to refute the argument that there is a large amount of non-$^3$H-leucine label in the ($^3$H-leu)-protein isolated in the present study.

Finally, the time course used in these turnover studies clearly prejudices the results to measure those proteins which turnover at a moderate rate ($t_{\frac{1}{2}}$ = 5 to 30 days). Proteins with shorter or longer $t_{\frac{1}{2}}$ values would not be measured.

Although the turnover of ($^3$H-leu)-protein in the crude mitochondrial fraction is inhibited in all brain regions by *d*-amphetamine, a complementary effect was not always observed in the microsomal or soluble fractions. Presently the mechanism(s) underlying the disassociation of drug effects between the various subcellular fractions is not clear. Almost any interpretation of the data would imply that amphetamine has a specific effect on the synthesis or metabolism of protein in the mitochondrial fraction and in some cases a distinctly separate effect on the synthesis or metabolism of microsomal or soluble protein.

## 11.9 GENERAL COMMENTS.

The reason for the use of $^3$H-leucine as the substrate in the pulse labeling studies was that the turnover kinetics had been extensively investigated by Von Hungen *et al.* (1968) and the validity of the technique established. As the ($^3$H-leu)-protein is metabolized, the free $^3$H-leucine made available is presumably immediately mixed with the endogenous leucine pool(s) and the resulting specific activity of the leucine pool is fairly low. Von Hungen *et al.* (1968) have determined by kinetic measurements that there is little re-utilization of label in the brain. It would seem unlikely that even drug administration could significantly affect re-utilization and thus the technique of using $^3$H-leucine is probably valid for measuring turnover in drug treated animals. However, when $^3$H-leucine is used for initial incorporation studies there are some serious problems that arise. Leucine is rapidly metabolized in the brain such that within 30 min after injection of labeled leucine the bulk of the label is no longer in leucine but rather in glutamate and glutamine (Clouet, 1970). However, Clouet (1970) also observed that only labeled leucine was incorporated into protein. The possibility must always be considered that drugs could have such an influence on leucine metabolism to either effect the rate of incorporation of labeled leucine into protein or to introduce non-leucine label into the proteins being investigated. Thus, it would be advisable for future studies to use some other labeled amino acid, e.g., $^3$H-lysine which is not metabolized by the brain.

# CHAPTER 12

## SUMMARY AND FINAL CONCLUSIONS

The purpose of this dissertation was to examine the chemical and behavioral aspects of amphetamine intoxication in rats and mice and, hopefully, establish causal relationship(s) between the behavioral and chemical parameters. Based on the results from several different yet related experiments, it appears that amphetamine intoxication causes the animal to respond to even small challenging doses of *d*-amphetamine with enhanced stereotyped behavior. The increase in exploratory activity observed after the acute administration of low doses of *d*-amphetamine in normal animals is apparently a very labile behavioral response which can be easily suppressed.

One of the reasons for the shift in behavior is that the DA to NE receptor activity ratio increases. It was established in Chapter 4 that even at doses of amphetamine which do not cause stereotyped behaviors there is an activation of the neostriatal DA neurons. In fact, it appears that activation of DA as well as NE neurons is required for amphetamine-induced increase in exploratory activity. When small or large doses of amphetamine are administered chronically, the apparent effectiveness of amphetamine to produce dopaminergic related behaviors, such as stereotyped activity, is greatly enhanced. This phenomenon is probably the result of both direct and indirect drug effects. One major indirect effect is related to the fact that in certain species, notably the rat, amphetamine is metabolized to the noradrenergic false transmitter and weak sympathomimetic, p-hydroxynorephedrine (PONE). When 10 μg of PONE is administered intracisternally, a technique whereby only a non-significant amount of the PONE reaches the neostriatum, the animal's responsiveness to subsequent challenging doses of *d*-amphetamine is greatly altered. In PONE pretreated animals an amphetamine challenge of 1 or 3 mg/kg i.p. primarily induces stereotyped behaviors rather than increasing stereotyped activity, which is the normal behavioral response. PONE does not produce this effect by altering NE metabolism, but rather by competing with NE at the receptor site and thus indirectly increasing the DA to NE receptor activity ratio.

There is some evidence that amphetamine intoxication may "activate" DA neurons by several mechanisms. The data in Chapter 6 suggest that intoxication increases DA synthesis and in the mouse brain causes the newly synthesized DA to be more readily released by challenging doses of *d*-amphetamine. The data in Chapter 7 support the results in Chapter 6 in terms of the effect of intoxication on the release of newly synthesized DA. Finally, Dr. Tseng in Dr. Loh's laboratory has preliminary evidence indicating that in rats the DA "receptor" is sensitized by chronic intoxication.

The question now arises as to whether or not the present data provide suitable information for devising new treatment programs to deal with the adverse behavioral effects observed clinically in the chronic amphetamine abuser? Presently, treatment primarily consists of administering the potent DA "receptor" blocking neuroleptics, e.g., chlorpromazine or haloperidol. However, some of the neuroleptics, notably chlorpromazine, also have marked effects on noradrenergic receptors.

This action would be undesirable since, based on the data in this study, treatment should be designed to increase NE receptor activity and decrease DA receptor activity. Thus, haloperidol would be predicted to be superior to chlorpromazine or thiothixene, since haloperidol has less adrenergic blocking actions. In combination with the neuroleptic, it would seem advisable to administer desipramine, an antidepressant which which blocks the reuptake of NE but not DA. Thus, the combination of haloperidol and desipramine should be effective in the treatment of the adverse behavioral effects including paranoid schizophrenia resulting from acute or chronic amphetamine intoxication.

Amphetamine, like many other psychoactive drugs, has a pronounced effect on REM sleep. Oswald and Thacore (1963) have observed that amphetamine decreases REM sleep, tolerance develops to this effect and upon drug withdrawal there is a marked REM rebound. Readministration of amphetamine blocks the REM rebound. In addition to REM rebound, withdrawal of amphetamine from the chronically dependent person results in marked depression (Watson *et al.*, 1972). Watson *et al.* (1972) have observed that withdrawal depression generally disappears within 2 to 3 days. This time course temporally coincides with the disappearance of PONE (Chapter 5). As discussed by Weil-Malherbe (1967), depression is usually associated with a decrease in NE receptor activity and in the context of the present study it was shown that PONE decreases NE receptor activity. The more persistent, albeit non-specific sign of amphetamine withdrawal is the REM rebound phenomenon. Based on the data of Jouvet (1969), conditions which enhance NE receptor activity should enhance REM sleep. The data in Chapter 7 demonstrate that after PONE has disappeared from the brain, there is still a marked increase in the release of newly synthesized NE (and DA) in the chronically intoxicated animal. At this time it is not clear if different or the same noradrenergic neurons are involved in REM sleep and depression. If they are the same, then clearly the increase in REM sleep cannot be initiated by the increase in NE release. If different noradrenergic neurons are involved, then the increase in NE release may be one of the pertinent underlying biochemical mechanisms of the REM rebound phenomenon. Regardless of which of the above situations proves correct, the increase in NE release will probably at least serve to amplify and to maintain the rebound effect.

Studies were conducted to determine if this change in release resulted from a modification of some nerve ending membrane constituent or constituents. Based in part on the data presented in Chapter 8, it appeared that an increase in release could result from a decrease in phosphatidylcholine turnover. Although amphetamine did decrease turnover in most subcellular fractions, no decrease in turnover was observed in the synaptic plasma membrane fractions. Even though this attempt to correlate a change in synaptic function with a modification in the functionality of a membrane constituent failed, I believe this is a prototype of the necessary future studies that will be required to fully understand both the acute and chronic mechanisms of drug action.

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# CURRICULUM VITAE

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## PUBLICATIONS

### A. Abstracts:

1. Hitzemann, R. J., Craves, F. B., Loh, H. H. and Domino, E. F.: Brain biogenic amines and motor activity following *d*-amphetamine pellet implantation in the mouse. Pharmacologist 11(2):255, 1969.
2. Hitzemann, R. J., Loh, H. H. and Domino, E. F.: Effects of *d*-amphetamine and p-methoxyamphetamine on adrenergic and serotonergic metabolism. Pharmacologist 12(2):3, 1970.
3. Tseng, L. F., Hitzemann, R. J. and Loh, H. H.: Comparative effects of *dℓ*-p-methoxyamphetamine and *d*-amphetamine on central catecholamines and locomotor activity. Pharmacologist, 1973.

### B. Proceedings:

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2. Hitzemann, R. J. and Loh, H. H.: The role of adrenergic mechanisms in amphetamine tolerance. NAS-NRC Committee on Problems of Drug Dependence, 2:1585-1606, 1971.

3. Hitzemann, R. J., Loh, H. H., Ho, A. and Way, E. L.: Effect of acute and chronic morphinization on cerebral $^{14}$C-tyrosine metabolism. I. Proc. Western Pharmacol. Soc., 14:91-94, 1971.

4. Hitzemann, R. J., Loh, H. H., Ho, A. K. S. and Way, E. L.: Effect of acute and chronic morphinization on cerebral $^{14}$C-tyrosine metabolism. II. Proc. Western Pharmacol. Soc., 14:91-94, 1971.

5. Hitzemann, R. J. and Loh, H. H.: Tolerance to amphetamine-induced psychomotor stimulation in mice. Proc. Western Pharmacol. Soc., 15:110, 1972.

6. Hitzemann, R. J. and Loh, H. H.: Effect of chronic morphine treatment on synaptic plasma membrane protein synthesis. NAS-NRC Committee on Problems of Drug Dependence, Mexico City, 1974.

### C. Full Papers:

1. Ho, A. K. S., Loh, H. H., Craves, F., Hitzemann, R. J. and Gershon, S.: Effects of lithium on brain biogenic amines turnover. Europ. J. Pharmacol. 10:72, 1970.

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4. Hitzemann, R. J. and Loh, H. H.: Effect of scopalamine on the conversion of $^{14}$C-tyrosine to $^{14}$C-catecholamine in the CNS. Pharmacology 8:368, 1972.

5. Hitzemann, R. J., Ho, I. K. and Loh, H. H.: Narcotic tolerance, dependence and serotonin turnover. Science 178:645, 1972.

6. Hitzemann, R. J., Loh, H. H. and Domino, E. F.: Effect of phencyclidine on the accumulation of $^{14}$C-catecholamine from $^{14}$C-tyrosine. Arch. Intl. Pharmacodyn. Ther. 202:252, 1973.

7. Loh, H. H., Hitzemann, R. J. and Way, E. L.: Effect of acute morphine administration on brain catecholamine metabolism. Life Science 12:33, 1973.

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